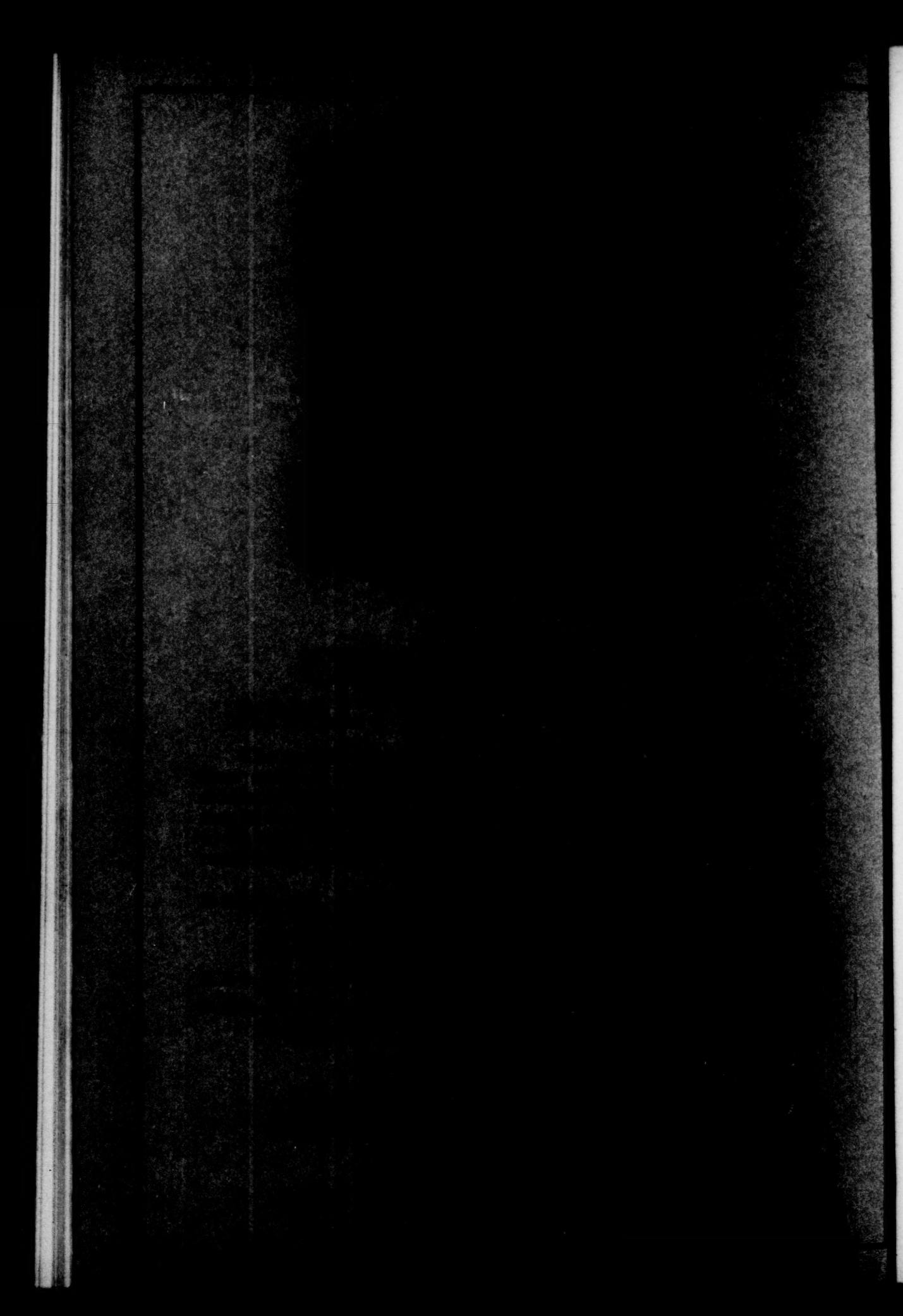

THE INFLUENCE OF ALKALI AND ALKALINE EARTH SALTS UPON THE RATE OF SOLUTION OF CASEIN BY SODIUM HYDROXIDE.

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One of the writers[1] has previously shown that when casein is shaken or stirred up in an alkaline solution, it dissolves rapidly at first, but later with slowness, and the relation between the time of stirring and the amount of casein dissolved obeys very accurately the relation $x = Kt^m$, where x is the number of grams of casein dissolved, t is the time, and K and m are constants which depend upon the concentration and kind of alkaline solution employed as solvent, and upon the total mass of casein present in the mixture. It appeared of interest to ascertain whether or not this relation held good in the presence of alkali or alkaline earth salts in the solvent, since these have a great influence upon the swelling and also upon the coagulation of colloidal substances, and, if the same relation exists when such solvent mixtures are employed, what influence these salts have upon the values of K and m. Accordingly the following experiments were undertaken.

The casein which was employed was Kahlbaum's casein *nach Hammersten*, specially purified by washing in distilled water, absolute alcohol, and ether according to the method described in a previous communication.[2]

The method of investigation which we adopted was that described in the previous paper;[2] that is, 100 cc. of the solvent were

[1] Robertson, T. B., *J. Phys. Chem.*, 1910, xiv, 377; *J. Biol. Chem.*, 1913, xiv, 237; *Arch. ges. Physiol.*, 1913, clii, 524.

[2] Robertson, *J. Phys. Chem.*, 1910, xiv, 377, 528.

placed in a 400 cc. beaker of squat form and agitated by a flattened glass rod which was bent at right angles, the plane of the horizontal arm being somewhat inclined to the vertical, so as to communicate an upward thrust to the rotating liquid. The stirrer was rotated at an approximately constant rate by a small electric motor. While stirring, 5 gm. of casein were dropped into the fluid. At stated intervals samples of the mixture were almost instantaneously extracted by means of a 10 cc. pipette which was provided with a rubber bulb. The samples were then very rapidly filtered through lightly packed glass wool. Using a Pulfrich refractometer, the angle of total reflection (i) of the filtrate from each sample was measured and its refractive index thus determined. Denoting the refractive index of the filtrates from any given sample by n and that of the pure solvent by n_1, the quotient $\frac{n-n_1}{0.00152}$ is the number of grams of casein dissolved in 100 cc. of the solvent at the moment when the sample was extracted.[3]

For the standard solvent we used 0.016 N sodium hydroxide solution. The salts employed were lithium, potassium, and sodium chlorides in the concentrations of 0.5 N, N, and 2 N and calcium, strontium, and barium chlorides at the concentrations of 0.005 N, 0.01 N, and 0.05 N.

Though it was previously proved by one of the writers[2] that temperatures lying between 20° and 36°C. do not affect the rate of solution of casein in solutions of the hydroxides of the alkalies, in these experiments we maintained a fairly constant temperature, placing the stirring machine in a small double walled room warmed by an electric hot plate set at "Low." The temperature through the experiments lay between 22° and 24°C. The temperature during the progress of each experiment is given. In the column headed "Calculated" are given the values of x calculated from the above formula, the constants K and m being determined from the results of the observations, employing for this purpose the form $\log x = \log K + m \log t$. The results obtained are as follows.

[3] Robertson, *J. Phys. Chem.*, 1909, xiii, 469.

TABLE I.

t	i	n	Casein in 100 cc. of solvent.		Δ (Difference.)
			Observed.	Calculated.	
Solvent: 0.016 N NaOH. i = 68.05. n_1 = 1.329250. Temperature: 23.5 − 24.0°C. K = 2.371. m = 0.151.					
min.			*gm.*	*gm.*	*gm.*
5	67.07	1.333714	2.98	3.02	+0.04
10	66.57	1.334497	3.45	3.36	−0.09
30	66.45	1.335445	4.08	3.96	−0.08
60	66.38	1.335990	4.43	4.40	−0.03
120	66.32	1.336470	4.75	4.91	+0.16
					$\Sigma\Delta$ = ±0.00
Solvent: 0.016 N NaOH + 0.5 N LiCl. i = 66.45. n_1 = 1.335445. Temperature: 22.5 − 23.0°C. K = 1.640. m = 0.216.					
5	66.01	1.338950	2.31	2.32	+0.01
10	65.53	1.339597	2.73	2.70	−0.03
30	65.40	1.340650	3.42	3.42	±0.00
60	65.30	1.341470	3.96	3.97	+0.01
120	65.18	1.342454	4.61	4.61	−0.00
					$\Sigma\Delta$ = −0.01
Solvent: 0.016 N NaOH + 1.0 N LiCl. i = 65.36. n_1 = 1.340778. Temperature: 22.5 − 23.0°C. K = 1.471. m = 0.223.					
5	64.58	1.344106	2.19	2.11	−0.08
10	64.53	1.344521	2.46	2.47	+0.01
30	64.41	1.345526	3.12	3.14	+0.02
60	64.32	1.346274	3.62	3.67	+0.05
120	64.20	1.347280	4.28	4.28	±0.00
					$\Sigma\Delta$ = ±0.00
Solvent: 0.016 N NaOH + 2.0 N LiCl. i = 63.33. n_1 = 1.351292. Temperature: 22.5 − 23.0°C. K = 0.977. m = 0.223.					
5	63.10	1.353270	1.30	1.40	+0.10
10	63.05	1.353705	1.59	1.63	+0.04
30	62.55	1.354575	2.16	2.09	−0.07
60	62.49	1.355097	2.50	2.43	−0.07
120	62.43	1.355619	2.85	2.84	−0.01
					$\Sigma\Delta$ = −0.01

TABLE I—*Continued.*

t	i	n	Casein in 100 cc. of solvent. Observed.	Casein in 100 cc. of solvent. Calculated.	Δ (Difference.)
Solvent: 0.016 N NaOH + 0.5 N NaCl. i = 67.05. n_1 = 1.333870. Temperature: 22.0 − 23.0°C. K = 1.397. m = 0.225.					
min.			*gm.*	*gm.*	*gm.*
5	66.26	1.336950	2.03	2.01	−0.02
10	66.20	1.337430	2.34	2.35	+0.01
30	66.08	1.338390	2.97	3.00	+0.03
60	65.57	1.339273	3.55	3.51	−0.04
120	65.47	1.340083	4.09	4.10	+0.01
					ΣΔ = −0.01
Solvent: 0.016 N NaOH + 1.0 N NaCl. i = 66.06. n_1 = 1.338550. Temperature: 22.0 − 23.0°C. K = 1.122. m = 0.228.					
5	66.35	1.341060	1.65	1.62	−0.03
10	65.30	1.341470	1.92	1.90	−0.02
30	65.21	1.342208	2.41	2.44	+0.03
60	65.13	1.342864	2.84	2.85	+0.01
120	65.04	1.343608	3.33	3.34	+0.01
					ΣΔ = ±0.00
Solvent: 0.016 N NaOH + 1.0 N KCl. i = 65.56. n_1 = 1.339354. Temperature: 22.5 − 23.0°C. K = 1.263. m = 0.221.					
5	65.22	1.342126	1.82	1.80	−0.02
10	65.17	1.342536	2.09	2.10	+0.01
30	65.07	1.343359	2.63	2.68	+0.05
60	64.58	1.344106	3.13	3.12	−0.01
120	64.48	1.344938	3.67	3.64	−0.03
					ΣΔ = ±0.00
Solvent: 0.016 N NaOH + 2.0 N KCl. i = 64.10. n_1 = 1.348130. Temperature: 22.5 − 23.0°C. K = 0.446. m = 0.243.					
5	63.58	1.349150	0.67	0.66	−0.01
10	63.56	1.349320	0.78	0.78	±0.00
30	63.52	1.349830	1.01	1.02	+0.01
60	63.49	1.349916	1.18	1.21	+0.03
120	63.44	1.350346	1.46	1.43	−0.03
					ΣΔ = ±0.00

TABLE I—*Continued.*

t	i	n	Casein in 100 cc. of solvent.		Δ (Difference.)
			Observed.	Calculated.	
Solvent: 0.016 N NaOH + 2.0 N NaCl. i = 64.20. n_1 = 1.347280. Temperature: 22.0 − 23.0°C. K = 0.363. m = 0.270.					
min.			*gm.*	*gm.*	*gm.*
5	64.10	1.348130	0.56	0.56	±0.00
10	64.08	1.348300	0.67	0.68	+0.01
30	64.04	1.348640	0.89	0.91	+0.02
60	64.00	1.348980	1.12	1.10	−0.02
120	63.56	1.349320	1.34	1.32	−0.02
					$\Sigma\Delta$ = −0.01
Solvent: 0.016 N NaOH + 0.5 N KCl. i = 66.56. n_1 = 1.334576. Temperature: 22.5 − 23.0°C. K = 1.677. m = 0.207.					
5	66.12	1.338070	2.30	2.34	+0.04
10	66.04	1.338710	2.72	2.70	−0.02
30	65.51	1.339759	3.41	3.39	−0.02
60	65.41	1.340569	3.94	3.91	−0.03
120	65.31	1.341388	4.48	4.52	+0.04
					$\Sigma\Delta$ = +0.01
Solvent: 0.016 N NaOH + 0.005 N $CaCl_2$. i = 67.59. n_1 = 1.329706. Temperature: 23.0 − 23.5°C. K = 1.728. m = 0.197.					
5	67.13	1.333246	2.33	2.37	+0.04
10	67.06	1.333792	2.69	2.72	+0.03
30	66.52	1.334892	3.41	3.38	−0.03
60	66.43	1.335603	3.88	3.87	−0.01
120	66.32	1.336470	4.45	4.44	−0.01
					$\Sigma\Delta$ = +0.02
Solvent: 0.016 N NaOH + 0.01 N $CaCl_2$. i = 67.58. n_1 = 1.329782. Temperature: 22.0 − 23.0°C. K = 1.365. m = 0.213.					
5	67.20	1.332700	1.92	1.92	±0.00
10	67.15	1.333090	2.18	2.23	+0.05
30	67.03	1.334006	2.78	2.82	+0.04
60	66.52	1.334892	3.36	3.37	+0.01
120	66.42	1.335682	3.88	3.78	−0.10
					$\Sigma\Delta$ = ±0.00

TABLE I—*Continued.*

t	i	n	Casein in 100 cc. of solvent. Observed.	Casein in 100 cc. of solvent. Calculated.	Δ (Difference.)
Solvent:* 0.016 N NaOH + 0.05 N $CaCl_2$. i = 67.55. n_1 = 1.330010. Temperature: 23.0 − 23.5°C. K = 0.459. m = 0.172.					
min.			*gm.*	*gm.*	*gm.*
5	67.49	1.330467	0.60	0.60	±0.00
10	67.48	1.330544	0.70	0.68	−0.02
30	67.47	1.330660	0.85	0.82	−0.03
60	67.46	1.330698	0.91	0.93	+0.02
120	67.45	1.330775	1.01	1.05	+0.04
					ΣΔ = +0.01
Solvent: 0.016 N NaOH + 0.005 N $SrCl_2$. i = 67.56. n_1 = 1.329934. Temperature: 22.5 − 23.0°C. K = 1.641. m = 0.200.					
5	67.12	1.333324	2.23	2.27	+0.04
10	67.05	1.333870	2.59	2.60	+0.01
30	66.51	1.334971	3.31	3.24	−0.07
60	66.42	1.335682	3.78	3.72	−0.06
120	66.34	1.336310	4.19	4.27	+0.08
					ΣΔ = ±0.00
Solvent: 0.016 N NaOH + 0.01 N $SrCl_2$. i = 67.58. n_1 = 1.329782. Temperature: 22.0 − 23.0°C. K = 1.135. m = 0.233.					
5	67.24	1.332392	1.72	1.65	−0.07
10	67.19	1.332778	1.97	1.94	−0.03
30	67.09	1.333558	2.48	2.51	+0.03
60	67.01	1.334182	2.89	2.96	+0.07
120	66.50	1.335050	3.47	3.48	+0.01
					ΣΔ = +0.01
Solvent:* 0.016 N NaOH + 0.05 N $SrCl_2$. i = 67.45. n_1 = 1.330774. Temperature: 22.5 − 23.0°C. K = 0.462. m = 0.214.					
5	67.39	1.331237	0.61	0.65	+0.04
10	67.37	1.331391	0.81	0.76	−0.05
30	67.35	1.331545	1.01	0.96	−0.05
60	67.34	1.331622	1.12	1.11	−0.01
120	67.33	1.331699	1.22	1.29	+0.07
					ΣΔ = ±0.00

TABLE I—*Concluded.*

t	i	n	Casein in 100 cc. of solvent.		Δ (Difference.)
			Observed.	Calculated.	
Solvent: 0.016 N NaOH + 0.005 N $BaCl_2$. i = 67.58. n_1 = 1.329782. Temperature: 23.0 − 24.0°C. K = 1.660. m = 0.189.					
min.			*gm.*	*gm.*	*gm.*
5	67.14	1.333168	2.23	2.25	+0.02
10	67.07	1.333714	2.59	2.57	−0.02
30	66.55	1.334655	3.21	3.16	−0.05
60	66.47	1.335287	3.62	3.60	−0.02
120	66.39	1.335910	4.03	4.10	+0.07
					$\Sigma\Delta$ = ±0.00
Solvent: 0.016 N NaOH + 0.01 N $BaCl_2$. i = 67.59. n_1 = 1.329706. Temperature: 22.0 − 23.0°C. K = 0.842. m = 0.240.					
5	67.34	1.331622	1.26	1.24	−0.02
10	67.31	1.331853	1.41	1.46	+0.05
30	67.21	1.332623	1.92	1.91	−0.01
60	67.14	1.333168	2.28	2.25	−0.03
120	67.07	1.333714	2.64	2.66	+0.02
					$\Sigma\Delta$ = +0.01
Solvent:* 0.016 N NaOH + 0.05 N $BaCl_2$. i = 67.44. n_1 = 1.330852. Temperature: 23.0 − 24.0°C. K = 0.221. m = 0.243.					
5	67.41	1.331083	0.30	0.33	+0.03
10	67.40	1.331160	0.41	0.39	−0.02
30	67.39	1.331237	0.51	0.51	±0.00
60	67.38	1.331314	0.61	0.60	−0.01
120	67.37	1.331391	0.71	0.71	±0.00
					$\Sigma\Delta$ = ±0.00

* Owing to the cloudiness of the solution obtained, it was diluted with the same volume of the solvent. The amount of the casein dissolved in 100 cc. solvent was, therefore, calculated according to the formula $2 \times \frac{n - n_1}{0.00152}$.

The possible experimental error in the determination of the concentration of a casein solution by means of its refractive index is always 0.07 gm. per 100 cc. It will be seen from Table I that the observed and calculated values of x are very close and

the differences (Δ) between these values are hardly ever greater, usually considerably less, than the possible error in the determination of the concentration of the casein in the filtrate. It is, therefore, evident that the relation between the time of stirring and the amount of casein dissolved obeys the relation $x = Kt^m$ when alkali or alkaline earth salts are present in the sodium hydroxide solution employed as solvent. In each instance, however, the presence of the salt caused retardation of the rate of solution and this retardation (between the limits of concentration studied) was greater the greater the concentration of the salt.

In regard to their power of causing retardation of solution, there is a great difference between the salts of the alkalies and those of the alkaline earths. Alkali chlorides in 0.5 N concentration exert about the same retardation as alkaline earth chlorides in 0.005 N concentration. The power of the alkaline earth chlorides to retard the rate of solution of casein is, therefore, approximately 100 times greater than that of the chlorides of the alkalies. This remarkable quantitative disparity between the effects of alkaline earth chlorides and the chlorides of the alkalies is strikingly analogous to the familiar disparity of their effects upon living tissues. Now the fact that the law $x = Kt^m$, the time relationship which is characteristic of surface tension phenomena,[4] holds good in the presence of these salts shows that their effect upon the rate of solution of casein is primarily to be attributed to their effect upon the rate of penetration of the casein particles by the solvent. The possibility thus suggests itself that in the actions of salts upon living tissues we may also be dealing with the effects of alterations in penetrability, a view to which experiments of Osterhout[5] and Loeb and Beutner[6] afford substantial support.

The retarding effect of the salts employed increases in the following order: Li < K < Na < Ca < Sr < Ba.

From the fact that NaCl exerts only a slightly greater retarding influence than KCl although sodium hydroxide is the alkali

[4] Bell, J. M., and Cameron, F. K., *J. Phys. Chem.*, 1906, x, 658. Ostwald, W., and Goppelsroeder, F., *Z. kolloid. Chem., Suppl.*, 1908, ii, 20.

[5] Osterhout, W. J. V., *Science*, 1912, xxxv, 112.

[6] Loeb, J., and Beutner, R., *Biochem. Z.*, 1912, xli, 1.

employed as solvent, we may infer that the retarding effect of these salts cannot be wholly attributed to diminution of the dissociation of the hydroxide due to the presence of a salt with a common ion.

The magnitudes of the constants K and m are affected by the salts in opposite senses; and the value of K decreases as the concentration of salt increases, while that of m increases.

Differentiating the equation $x = Kt^m$ we find: $\frac{dx}{dt} = Kmt^{m-1}$ in other words, the product Km, which we may term the *coefficient of penetration* expresses the constant proportionality between the velocity of solution and an exponent (peculiar to each solvent) of the time during which the protein has been exposed to the action of the solvent.

Since the value of K decreases more rapidly than m increases with increasing concentration of salt, the effect of all the salts, within the limits of concentration employed, is to cause a decrease in the value of the coefficient of penetration, a decrease which is a definite function of the concentration of salt employed.

The relationship between the value of the coefficient of penetration and the concentration of salt added to the solvent may be defined by the interpolation equation $K_1m_1 - Km = \alpha c + \beta c^2$ where K_1m_1 is the coefficient of penetration for the pure solvent (0.016 N NaOH), Km is its value when a given salt has been added in concentration c, and α and β are constants dependent upon the nature of the added salt.

In Table II the observed values of the coefficient of penetration are compared with the values calculated from the equation; the values of the constants α and β being calculated by the method of least squares from all of the observations obtained with the given salt.

These results reveal another striking difference between the effects of the chlorides of the alkalies and of the alkaline earths upon the rate of penetration of the casein particles by the solvent, for whereas NaCl and KCl in increasing concentration decrease the rate of penetration with a *positive* acceleration, $CaCl_2$, $SrCl_2$, and $BaCl_2$ decrease the rate of penetration with a *negative* acceleration, which implies that at sufficiently high concentrations they would actually accelerate the penetration of

TABLE II.

Concentration.	Km		Difference.
	Found.	Calculated.	
LiCl. $K_1m_1 = 0.356$. $\alpha = -0.013$. $\beta = +0.041$.			
0.00	0.356	0.356	±0.000
0.50	0.354	0.352	−0.002
1.00	0.328	0.328	±0.000
2.00	0.218	0.218	±0.000
NaCl. $K_1m_1 = 0.356$. $\alpha = +0.066$. $\beta = +0.032$.			
0.00	0.356	0.356	±0.000
0.50	0.315	0.315	±0.000
1.00	0.258	0.258	±0.000
2.00	0.097	0.096	−0.001
KCl. $K_1m_1 = 0.356$. $\alpha = +0.030$. $\beta = +0.047$.			
0.00	0.356	0.356	±0.000
0.50	0.347	0.329	−0.018
1.00	0.279	0.279	±0.000
2.00	0.108	0.108	±0.000
$CaCl_2$. $K_1m_1 = 0.356$. $\alpha = +8.100$. $\beta = -12.250$.			
0.000	0.356	0.356	±0.000
0.005	0.340	0.346	+0.006
0.010	0.291	0.289	−0.002
0.050	0.251	0.248	−0.003
$SrCl_2$. $K_1m_1 = 0.356$. $\alpha = +10.200$. $\beta = -10.150$.			
0.000	0.356	0.356	±0.000
0.005	0.328	0.330	+0.002
0.010	0.264	0.264	±0.000
0.050	0.099	0.099	±0.000
$BaCl_2$. $K_1m_1 = 0.356$. $\alpha = +17.700$. $\beta = -23.400$.			
0.000	0.356	0.356	±0.000
0.005	0.314	0.326	+0.012
0.010	0.202	0.202	±0.000
0.050	0.054	0.054	±0.000

the casein by the solvent. The study of the effects of higher concentrations of alkaline earth chlorides upon the rate of penetration of casein is, however, complicated by a number of factors which tend to obscure these relationships. Investigations of these effects are in progress and will form the subject of a subsequent communication. Lithium chloride occupies an exceptional position among the salts studied, since with increasing concentration it increases the rate of penetration with *negative* acceleration. This implies that at a sufficiently low concentration (less than M/3) the presence of LiCl might be expected to accelerate the penetration of casein particles by 0.016 N NaOH. A simple calculation serves to show, however, that this effect even if present would be too small to be measurable by the technique employed.

CONCLUSIONS.

1. The relation between the time which has elapsed since the casein was introduced into the solvent and the amount of casein dissolved is expressed by the relation $x = Kt^m$ even in the presence of alkali or alkaline earth chlorides in the sodium hydroxide solution employed as solvent.

2. The presence of these salts decreases the rate of solution of casein by dilute sodium hydroxide. The retardation increases with the concentration of salt employed.

3. The alkaline earth chlorides in 0.005 N concentration retard the rate of solution of casein approximately to the same extent as 100 times this concentration of a chloride of an alkali.

4. The value of K decreases while that of m increases with increase of the concentration of the salts. The value of the product $K \times m$, which we term the coefficient of penetration, decreases with increasing concentrations of NaCl, KCl, $CaCl_2$, $SrCl_2$, or $BaCl_2$ between the limits of concentration employed, the acceleration of the decrease being positive in the cases of NaCl and KCl and negative in the cases of $CaCl_2$, $SrCl_2$, or $BaCl_2$. Lithium chloride increases the value of the coefficient of penetration, with a negative acceleration so that at concentrations lying above 0.33 N the algebraic sum of these opposite effects results in a decrease of the value of the coefficient of penetration.

STUDIES OF AUTOLYSIS.

IV. THE LATENT PERIOD IN AUTOLYSIS.

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In 1904 Lane-Claypon and Schryver[1] reported a latent period in autolysis amounting in the normal liver to about 4 hours. In the livers of fasting animals the latent period was absent or much shorter than in the well fed. If active trypsin preparations were introduced into the autolyzing liver mass, digestion began at once. These observations led the authors to the conclusion that in the living liver cell no free proteolytic enzymes existed; that the first stage of postmortem disintegration set free an active enzyme from a zymogen, and that the latent period represented, therefore, a survival period in the tissue. The authors further concluded that during fasting the zymogen was activated in some way, and thus autolysis intervened to allow the starving animal to utilize its own tissues for the development of energy by combustion, which otherwise would remain unavailable. The validity of this attractive hypothesis rests upon the rather limited observations reported in this one paper, and upon the interpretation of those observations. It has appeared to us worth while to examine further the early stages of autolysis of the liver in the hope that some new insight might be gained into the mechanism which starts and controls this important reaction. In the present paper we have made no attempt to test the differences between well fed and starving material, though both kinds are represented in our data. To a certain extent we have confirmed the findings of Lane-Claypon and Schryver, but our interpretation of the phenomenon is at variance with theirs. We have called attention to the great individual differences in appar-

[1] Lane-Claypon, J. E., and Schryver, S. B., *J. Physiol.*, 1904, xxxi, 169.

ently normal livers in a previous paper[2] and we have found that such differences are very apparent in the early stages of autolysis. Such differences we believe are undoubtedly associated with differences in the nutritional condition of the organ, and in a later paper we expect to present data upon this point.

Experiment I.—Three well fed rabbits were killed and bled. The livers were removed at once, ground to a fine pulp, divided equally, and each half was made up to 500 cc., with toluene water. 25 minutes after the death of the animal the initial samples were removed and the digests warmed quickly to 37°C. To one digest sufficient HCl was added to have made it a 0.02 N solution. Two sets of samples were taken, one precipitated by trichloroacetic acid as described in a previous paper,[2] the other coagulated by heat in the presence of KH_2PO_4. The samples from the acid digest were first treated with $CaCO_3$ to neutralize the mineral acidity, an excess of KH_2PO_4 was added, and the sample plunged into a boiling water bath. Boiling water was added till the volume approximated 90 cc. and the samples were kept at the boiling point 3 minutes after coagulation appeared to be complete. Control samples were coagulated in the same way. Heat-coagulated samples were cooled, made up to 100 cc., and filtered. The filtrate was analyzed for total nitrogen by the Kjeldahl method and for amino nitrogen by the Van Slyke micro method.

A survey of the figures shows: 1. The initial sampling is uncertain. The diffusion of water into the protein masses and the diffusion out of soluble nitrogenous compounds has not attained equilibrium. The initial samples are therefore subject to considerable error and their value must be interpreted in relation to the figures obtained in subsequent samples. We find the same irregularities in the initial values obtained by Schryver and we believe there is nothing significant in the low or high figures of the initial samples other than lack of equilibrium.

2. The non-coagulable nitrogen of the control shows a latent period of about 1 to 2 hours before unmistakable increase appears. In the acid digest, on the other hand, there is no latent period, or it is less than 30 minutes.

3. The Van Slyke method shows a latent period in the amino-acid appearance of 4 to 8 hours in the control, and of about 1 hour in the acid digest.

4. The trichloroacetic acid method shows increase of amino-

[2] Bradley, H. C., and Taylor, J., *J. Biol. Chem.*, 1916, xxv, 261.

TABLE I.

Rabbit Livers.

Time.	0	30 min.	60 min.	2 hrs.	4 hrs.	8 hrs.	27 hrs.	4 days.
Trichloroacetic acid method.								
Cc. 0.2 N amino-acid per 6.25 cc. tissue suspension.								
I. Control.....	0.25	0.30	0.30	0.30	**0.50**	0.60	0.70	0.80
II. " HCl.....	0.30	0.30	**0.40**	0.40	0.70	1.30	2.40	3.20
Non-coagulable N.								
Cc. 0.2 N NH_3 per 6.25 cc. tissue suspension.								
I.	1.75	1.60	**1.90**	2.00	2.15	2.20	2.30	2.45
II.....	1.80	**2.20**	2.20	3.20	3.30	4.90	6.30	8.60
Van Slyke method.								
Mg. N per 1 cc. filtrate or 0.25 cc. tissue suspension.								
I.....	0.076	0.060	0.060	0.060	**0.076**	0.082	0.087	0.114
II.....	0.054	0.049	**0.087**	0.103	0.157	0.211	0.477	0.604

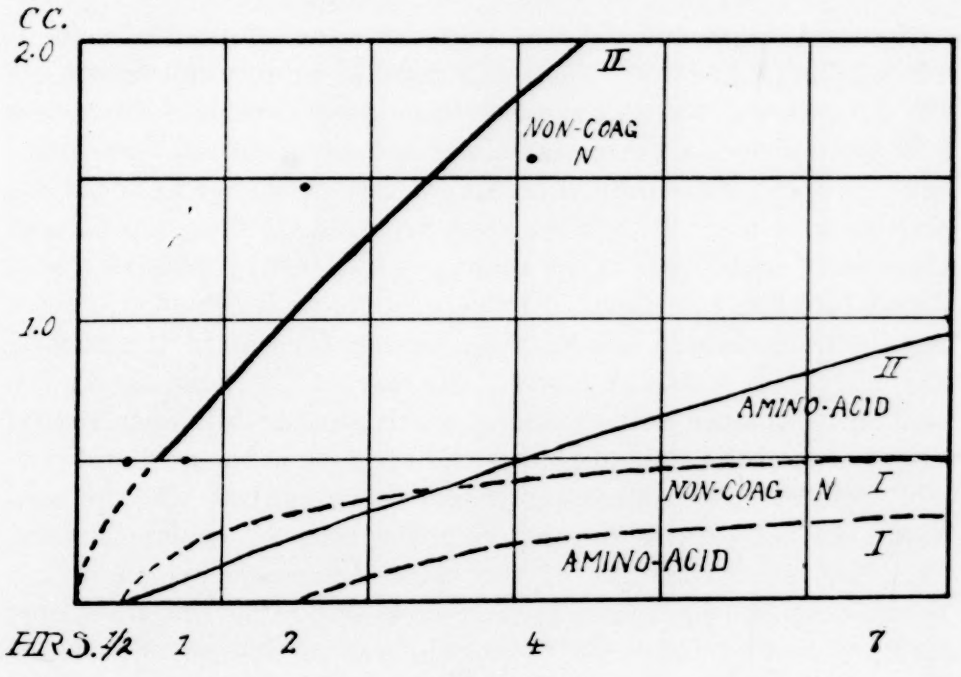

FIG. 1.

acids in the control after 2 to 4 hours, in the acid digest after 1 to 2 hours.

The figures and curves clearly indicate a lag between the appearance of increased non-coagulable nitrogen and the appearance of amino-acids. The lag would, therefore, appear to express the time required in progressive hydrolysis of native proteins to reach, first, a measurable increase of non-coagulable protein, and second, a measurable increase of amino-acids. If amino-acids are split off during the earlier stages of the reaction they are not measurable by the technique employed in this experiment. It should be noted that Schryver used heat coagulation with trichloroacetic acid present to throw down his undigested material; his figures therefore are comparable with those obtained by us with trichloroacetic acid precipitation. In this particular case our results agree very well with Schryver's. It is quite possible therefore that Schryver's latent period was nothing more significant than the time required between the onset of autolysis and the appearance of the final cleavage products.

In all cases the presence of acid cuts down the latent period, just as it has been shown to increase the rate and extent of hydrolysis in longer digestions. The acid digest reaches approximate equilibrium in 4 days, at four times the amino-acid level of the control (3.2 to 0.8). This difference in equilibrium represents the difference in the actual substratum available for the enzymes in the two digests. Such a difference in mass of substratum should cause proportionate differences in the rate of change at the start, and we find in fact that the time required to show amino-acid increase is about four times as long in the control as in the acid digest (4 hours to 1 hour). If we assume, with Schryver, that a certain time elapses before the proteases present in the control are activated (about 4 hours), whereas in the acid digest the enzyme is at once active, then a much greater difference should exist between the time of amino-acid increase in the acid and control digests. The differences between the control and the acid digest do not express differences in the time of enzyme activation, but are expressive of the same differences which obtain throughout the continued hydrolysis, determining rate of change and point of equilibrium. These differences appear to us to be indicative of different masses of substratum only.

In one of Schryver's experiments trypsin was added to a fresh liver digest. Hydrolysis was apparent at once, while in the control there was the usual 4 hour latent period. But to trypsin practically all the liver proteins are substratum, while to the liver proteases only a small fraction—about 25 per cent on the average—is available for the reaction. With this difference in the specificity of the two enzymes, and with no means of knowing the amount of trypsin added compared to the proteases already there, a comparison of these two digests is without significance and certainly does not prove Schryver's contention that the latent period represents the time elapsed before the enzyme becomes active.

Experiment II. The Amino-Acid Lag in Rapid Autolysis.—Liver obtained from an old horse and removed within a minute of the death of the animal, was sliced, chilled to −10°C., and kept frozen till ground to a snow and mixed with toluene water at 0°C. Samples were taken during a 10 minute interval, the time required to bring the digest to 37°C., acid was added, and sampling continued at short intervals. Van Slyke and Kjeldahl figures were obtained on the heat coagulation filtrates.

TABLE II.

Horse Liver.

Time.	Van Slyke method. Mg. N per 1 cc. filtrate or 0.25 cc. tissue suspension.	Non-coagulable nitrogen. Cc. 0.2 N NH_3 per 6.25 cc. tissue suspension.
min.		
0	0.095	1.70
10	0.111	1.70
12	0.106	**2.05**
15	**0.123**	2.30
20	0.128	2.60
25	0.128	3.00
30	0.128	3.10
35	0.145	3.20
40	0.145	3.30
50	0.159	3.60
60	0.162	3.70
70	0.173	3.85

In this case, we find immediate response to the addition of acid in the total nitrogen, with a delayed response in the amino-acid figures. Both curves rise most rapidly at the start, and are of

the logarithmic type. If acid is the activator of the enzymes, as Morse has recently maintained,[3] then at the moment of the addition of HCl the enzymes were completely active. Still we find the lag between early and late products of autolysis. In other words, ruling out any possible difference in the time of activation, we still find a typical latent period when we measure amino-acids. *In this case, then, it is certain that the latency of the reaction does not indicate activation,* but merely reaction lag, which is sufficient to account for Schryver's results.

Experiment III. The Effect of Added Substratum.—The same material was used as in Experiment II. Gelatin solution was added as an available foreign protein. It was boiled with $CaCO_3$ for several hours and filtered, thus insuring neutrality. The gelatin was found to digest at once with no clear indication of a latent period. Making due allowance for the errors of end-point in the titrations, there was undoubted increase in amino-acids within 60 minutes; in the control equally certain increases were not found until 4 to 8 hours had elapsed.

In this case, then, we have abolished the latent period, or cut it down largely, by a procedure which was designed to eliminate a possible activating action of acids. By simply increasing the mass of substratum, itself neutral, we have duplicated the effect on the early stages of autolysis produced by adding mineral acids. If the possibility of an acid activation latency can be abolished by increasing the mass of substratum, then the enzyme must either have been active already, or the gelatin activated it—an improbable alternative. It seems clear in this case that an acidity not above that of the normal living tissue is all that is necessary to demonstrate activity of the enzyme provided there is any appreciable amount of substratum present.

Experiment IV. The Effect of Reaction.—The material was the same as that used in Experiment II. Two sets of samples were taken as before. The Van Slyke figures are upon 1 cc. of filtrate and represent 0.25 cc. of the digest. The non-coagulable nitrogen figures are upon 25 cc. of the same filtrates and represent 6.25 cc. of the digest.

The early readings with the Van Slyke apparatus were all small and subject to rather large errors in our hands. They indicate but slight increase of the amino nitrogen until the 24 hour period.

[3] Morse, M., *J. Biol. Chem.*, 1916, xxiv, 163.

TABLE III.

Trichloroacetic Acid Method.

Cc. 0.2 N Amino-Acid per 6.25 Cc. Tissue Suspension.

Time.	0	15 min.	30 min.	60 min.	90 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.
I. Control...	0.40	0.50	0.50	0.50		0.55	0.50	0.60	0.65
II. Gelatin...	0.80	0.85	0.90	0.95	0.95	1.05	1.15	1.35	1.70

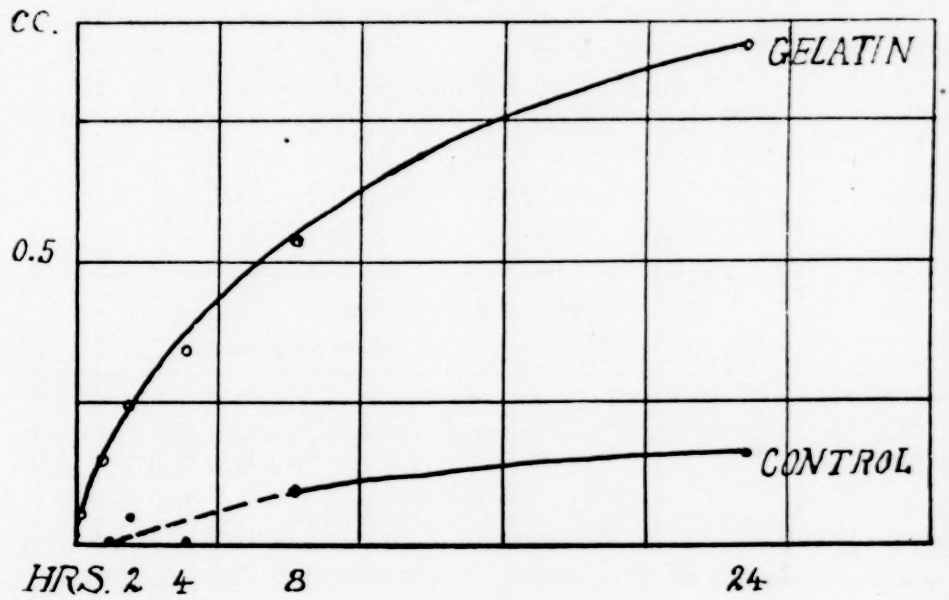

FIG. 2.

In the acid mixtures the increase occurs earlier. In the alkaline mixtures the figures are irregular, but digestion is slight until the 24 hour period, and is generally less as the bicarbonate is increased. The neutral phosphate also inhibits the digestion markedly.

In the trichloroacetic acid filtrates a clearly defined latent period is difficult to recognize. Some digestion appears to be under way by the end of an hour—assuming that the correct figure for the initial samples is about 0.45 cc. With acid present the lag is decreased in proportion to the amount added. In the alkaline samples there appears to be digestion about like that of the control, or somewhat less. The phosphate mixtures behave in the same way.

TABLE IV.

Content of digestion bottles.	0	15 min.	30 min.	60 min.	90 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.	3 days.
Van Slyke method.										
Mg. N per 1 cc. filtrate or 0.25 cc. tissue suspension.										
I. Control	0.07		0.07	0.07		0.08		0.07	**0.08**	0.11
II. " HCl to 0.01 N.	0.07	0.07	0.07	**0.09**	0.11	0.11	0.13	0.19	0.28	0.35
III. " " " 0.02 "	0.07		0.07	**0.11**	0.15		0.23	0.24	0.43	0.60
IV. " " " 0.04 "	0.07	0.07	0.06	**0.12**	0.19	0.19	0.29	0.40	0.56	0.78
V. " $NaHCO_3$ 0.5 gm	0.07			0.08				0.07	**0.08**	0.09
VI. " " 1.0 "								0.08	0.07	**0.09**
VII. " " 2.0 "	0.06					0.06	0.07	0.08	0.07	0.07
VIII. " K_2HPO_4 5.0 "	0.08						0.07	0.09		0.07
IX. " " 2.0 "	0.06							0.07	0.06	
Non-coagulable nitrogen.										
Cc. 0.2 N NH_3 per 6.25 cc. tissue suspension.										
I. Control	2.4		1.6	1.6	1.6	**1.7**	**1.9**	1.8	2.1	2.7
II. " HCl to 0.01 N.	2.2	1.7	**1.9**	2.0	2.2	2.2	2.7	3.3	3.9	5.4
III. " " " 0.02 "	2.2		**2.3**	2.6	2.9	3.3	4.0	5.4	6.7	8.8
IV. " " " 0.04 "	2.2	**2.7**	3.0	3.7	4.5	4.9	6.0	7.6	8.7	10.7
V. " $NaHCO_3$ 0.5 gm	1.5					1.6	**1.7**	1.7	1.8	2.2
VI. " " 1.0 "	1.9					**1.7**		1.7	1.8	2.2
VII. " " 2.0 "	1.6						**1.9**	2.0	1.9	2.3
VIII. " K_2HPO_4 5.0 "	1.8					1.6	1.7	1.7	**1.8**	2.3
IX. " " 2.0 "	1.7					1.7	1.7	1.7	**1.8**	2.5
Trichloroacetic acid method.										
Cc. 0.2 N amino-acid per 6.25 cc. tissue suspension.										
I. Control	0.25		**0.45**	**0.50**	0.50	0.55		0.60	0.80	1.00
II. " HCl to 0.01 N.	0.35	0.40	**0.45**	**0.55**	0.60	0.65	0.90	1.25	1.75	
III. " " " 0.02 "	0.40	0.40	**0.45**	**0.65**	0.80	0.80	1.10	1.60	2.45	
IV. " " " 0.04 "	0.50	**0.45**	**0.55**	0.75	0.95	1.10	1.50	2.15	2.80	4.40
V. " $NaHCO_3$ 0.5 gm	0.40		**0.45**	**0.45**		0.60			0.70	0.85
VI. " " 1.0 "	0.35		0.45	0.40		**0.65**		0.60	0.80	0.90
VII. " " 2.0 "	0.35		0.40	0.40			**0.55**		0.60	0.80
VIII. " K_2HPO_4 5.0 "	0.40		**0.45**	**0.45**			0.50	0.50	0.70	0.80
IX. " " 2.0 "	0.40		0.45	0.40	**0.50**	0.60	0.60	0.60	0.60	0.80

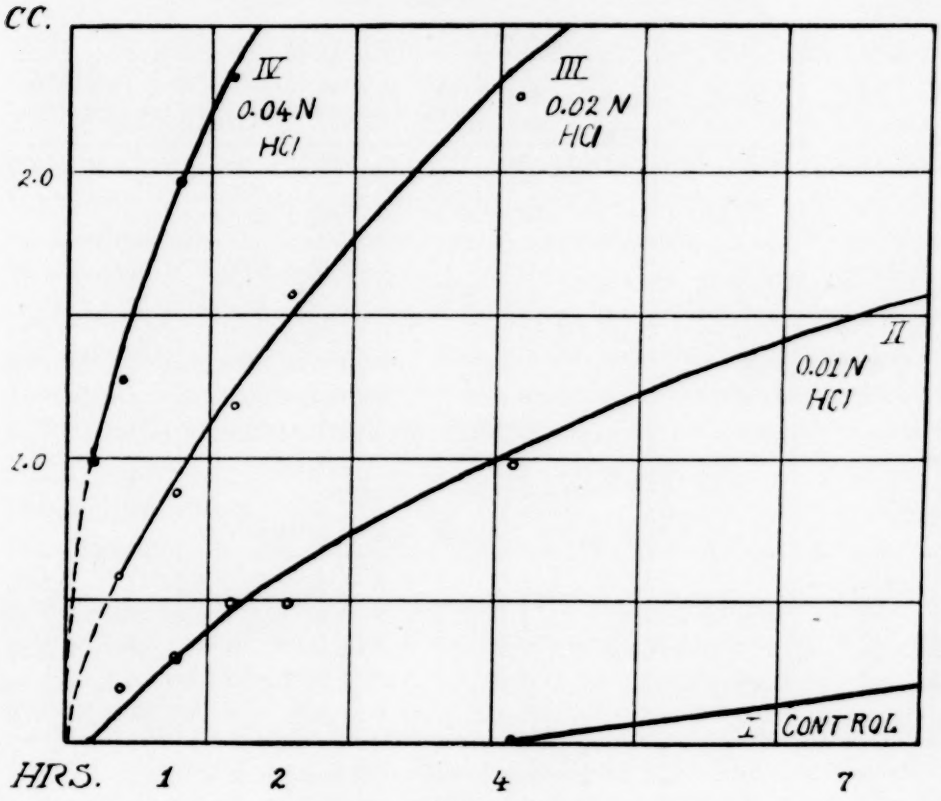

Fig. 3.

Non-coagulable nitrogen shows a lag in the control of about 2 to 4 hours before digestion is definitely seen. Increasing acidity cuts down this lag so that the most acid sample is clearly moving within 15 minutes. The figures on the alkaline or salt samples are irregular but appear to indicate a lag of 4 to 8 hours before digestion is definitely under way.

Despite the rather large irregularities of our figures in this series, which we believe represent inevitable irregularities of sampling, of equilibrium by diffusion, and of heat coagulation, the latent period appears to be shortened by acids, and lengthened by neutrality or diminished acidity. None of the digests were permanently alkaline to litmus.

Experiment V.—Liver obtained from a healthy well nourished calf was frozen and ground to a snow. All digests were sampled and then rapidly warmed to 37°C.

TABLE V.

Content of digestion bottles.	0	15 min.	30 min.	60 min.	90 min.	2 hrs.	4 hrs.	8 hrs.	24 hrs.
Van Slyke method. Mg. N per 1 cc. filtrate or 0.25 cc. tissue suspension.									
I. Control	0.04		0.04	**0.05**	0.07	0.07	0.07	0.08	0.09
II. " HCl to 0.04 N	0.04	**0.06**	0.08	0.09	0.10	0.12	0.14	0.17	0.25
III. " 0.25 gm. $NaHCO_3$	0.04		0.06	**0.05**	0.06	0.07	0.07		0.12
IV. " 0.50 " "	0.04		0.06	0.06	**0.05**	0.06	0.07	0.08	0.08
V. " 1.00 " "	0.04		0.04	0.06	**0.05**	0.06		0.06	0.07
VI. " gelatin	0.10		**0.12**	0.13	0.15	0.16	0.18	0.20	0.25
Non-coagulable nitrogen. Cc. 0.2 N NH_3 per 6.25 cc. tissue suspension.									
I. Control	1.7		**1.8**	1.8	2.05	2.10	2.15		2.30
II. " HCl to 0.04 N	1.7	**2.4**	2.8	2.95	3.00	3.60	4.00	4.30	5.30
III. " 0.25 gm. $NaHCO_3$	1.7		1.70	**1.90**		1.90	1.95	2.00	2.20
IV. " 0.50 " "	1.8		1.70	1.75	1.80	**1.75**	1.95	1.90	2.10
V. " 1.00 " "	1.70		1.60	1.65		1.70	**1.80**	1.80	2.00
Trichloroacetic acid method. Cc. 0.2 N amino-acid per 6.25 cc. tissue suspension.									
I. Control	0.45		0.45	0.45	**0.55**	0.60	0.65	0.70	0.85
II. " HCl to 0.04 N	0.40	**0.45**	**0.50**	0.55	0.70	0.70	0.80	1.05	1.50
III. " 0.25 gm. $NaHCO_3$	0.50			0.55	0.50	**0.55**	0.60	0.70	0.75
IV. " 0.50 " "	0.45			0.50		**0.55**	0.60	0.65	0.70
V. " 1.00 " "	0.45			0.50		0.50	**0.55**	0.60	0.65
VI. " gelatin	1.10		**1.20**	1.25	1.35	1.40	1.50	1.70	2.30

1. The non-coagulable nitrogen of the control appears rapidly, increasing within 90 minutes or less. The amino-acid nitrogen parallels the non-coagulable nitrogen in this case.

2. Acidity cuts down the latent periods. A lag is shown between the non-coagulable and amino-acid nitrogen by the trichloroacetic acid method.

3. In the presence of $NaHCO_3$ the non-coagulable nitrogen increase is delayed from 2 to 8 hours. On the other hand, the Van Slyke figures are irregular and show only a doubtful delay over the control until the 24 hour sample. The trichloroacetic acid figures show a more distinct delay in amino-acid production and a decided inhibition of the digestion all the way through.

4. The addition of neutral gelatin gives a reaction curve almost identical with the curve of the acid digest (in both trichloroacetic acid and Van Slyke methods). The experiment is in the nature of a coincidence, where the amount of substratum produced by the addition of acid was identical with the increase due to neutral gelatin. It gives strong confirmation to the belief that the question of activation of an enzyme is subsidiary in the mechanism which starts and controls autolysis and that the determinative factor is the amount of substratum present and the conditions which modify that amount in the living cell.

It was of interest to note that the glycogen turbidity of the control filtrates persisted even after 24 hours, though with constant diminution. In the acid samples and in the gelatin digest the glycogen of the filtrates disappeared much more rapidly.

Experiment VI.—Liver obtained from a 5 year old horse was removed within 2 minutes of the death of the animal and frozen at once.

TABLE VI.

Trichloroacetic Acid Method.

Cc. 0.2 N Amino-Acid per 6.25 Cc. Tissue Suspension.

	0	15 min.	30 min.	45 min.	60 min.	90 min.	2 hrs.	4 hrs.	8 hrs.	26 hrs.	3 days.
I. Control	0.30		0.35		0.45	0.45	0.55	0.60	0.60	0.65	1.00
II. " gelatin	0.65	0.60	0.60	0.60	0.70	0.80	0.85	0.95	1.05	1.30	2.25
III. " 0.5 gm. $NaHCO_3$	0.30		0.35		0.35	0.35	0.40	0.40	0.50	0.55	0.65
IV. Control 0.5 gm. $NaHCO_3$ gelatin	0.70	0.60	0.70	0.60	0.60	0.70	0.70	0.90	0.95	1.10	1.50
V. Control HCl to 0.04 N.	0.30	0.65	0.70	0.80	0.70	1.00	1.15	1.45	1.80	2.60	4.00
VI. " 1.0 gm. $NaHCO_3$	0.30		0.40		0.35	0.35	0.45	0.45	0.45	0.55	0.60

1. The control shows a latent period of about 60 to 90 minutes.

2. Gelatin shows digestion within 60 minutes.

3. Acid causes digestion within 15 minutes, far more rapid and extensive in this case than where gelatin was present. Though the same amounts of gelatin and acid were used as in Experiment V, the conditions are far from coincident in this liver.

4. Bicarbonate increases the latent period and the autolysis is very slight even in 3 days.

5. Gelatin added to a bicarbonate mixture reduces its latent period.

An analysis of the figures beginning with the 8 hour samples shows that gelatin is digesting in both normal and salt mixtures. At the end of 8 hours the digestion rate is indistinguishable, but in 26 hours the digestion of gelatin is evidently slower in the salt mixture. The 3 day sample also shows a certain amount of inhibition of the gelatin digestion. These figures bear out the findings reported in a previous paper[3] where the digestion of gelatin was found to be inhibited by alkalies in long continued autolysis, but where the inhibition was less than upon the digestion of liver proteins.

TABLE VII.

Analysis of Figures of Table VI.

Time.	8 hrs.		26 hrs.		3 days.	
	Gain.	Digestion of gelatin.	Gain.	Digestion of gelatin.	Gain.	Digestion of gelatin.
I.....	0.25		0.30		0.65	
II.....	0.45	0.20	0.70	0.40	1.60	0.95
III.....	0.15		0.20		0.30	
IV.....	0.35	0.20	0.50	0.30	0.90	0.60

These preliminary studies of the early stages of autolysis warrant the following conclusions:

1. The time required for the appearance of measurable amounts of amino-acids is greater than that required for the increase in non-coagulable nitrogen. The latent period reported by Lane-Claypon and Schryver is to be explained, therefore, as the lag between the initial stages of proteolysis and the final products.

2. The addition of the optimum amount of acid may reduce the lag to a very few minutes, from the usual 4 hours. At the same time the increase of non-coagulable nitrogen may be too rapid to show any latent period.

3. The addition of neutral gelatin in proper amount to a fresh autolyzing liver may reduce the latent period identically with acid. In this case the enzyme is proven to be active in the neutral or amphoteric liver material before any appreciable amount of acid has been formed in the mixture.

4. Since gelatin can hardly be an activating agent, and since its effect on the digest as a whole is identical with that of increased acidity, confirmation is found for the assumption that the effect of acid on liver material is to increase the mass of substratum in the mixture.

5. There is nothing in the data presented here which suggests the activation of an enzyme as an important determinative step in autolysis. If the enzyme is activated it takes place at a H^+ level such as is found in the living tissue. Furthermore, none of the curves show any indication of an autocatalytic phase in the early stages of the reaction, such as Morse assumed,[3] due to activation of the enzyme by developing acidity.

Length of the latent period, rate of proteolysis as measured by concentration of products, and final equilibrium of the digestion all appear to depend on the mass of substratum available for hydrolysis by the proteases present normally in the liver cell.

CHEMICAL STIMULATION OF NERVES.*

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(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

(Received for publication, May 12, 1916.)

I.

In 1899 Loeb[1] stated that electrolytes cause twitchings of muscles, while non-electrolytes have no such effect. But not all electrolytes were equally efficient as stimulants. The sodium salts seemed the best adapted for this purpose and their efficiency varied considerably with the nature of the anion, as the following list shows.[2]

TABLE I.

Salt.	Minimal stimulating concentration.	Salt.	Minimal stimulating concentration.
NaCl	M/16	Na_2 succinate	M/16
NaBr	M/16–M/32	Na_2SO_4	M/32
NaI	M/32	$NaHCO_3$	M/16–M/32
Na acetate	M/32–M/64	Na_2 oxalate	M/260
NaF	M/64–M/96	Na_2HPO_4	M/128
Na formate	M/80–M/160	Na_3 citrate	M/200

Since Ca inhibits the twitchings caused by NaCl, as Ringer first observed, Loeb suggested that the higher stimulating effect of oxalates, fluorides, and phosphates might be due to a precipitation of Ca in the tissues, but that this could not account for the high stimulating effect of certain other salts like Na formate.

In his studies on the chemical stimulation of nerves Mathews[3]

* These experiments were commenced in 1912 and completed during the year 1915–16. On account of the war it was impossible to submit the paper with the more recent results to Dr. Ewald before publication.

[1] Loeb, J., Festschrift f. Prof. Fick, 1899, p. 101.

[2] Loeb, *Arch. ges. Physiol.*, 1902, xci, 248.

[3] Mathews, A. P., *Am. J. Physiol.*, 1904, xi, 455.

found similar stimulating values for the sodium salts upon the motor nerve (sciatic of the frog).

TABLE II.

Salt.	Minimal stimulating concentration.	Salt.	Minimal stimulating concentration.
NaCl	M/12	Na_2SO_4	M/32
NaBr	M/12	$NaHCO_3$	M/5
NaI	M/12	Na_2 oxalate	M/32
Na acetate	M/12	Na_2HPO_4	M/35
NaOH	M/20	Na_3 citrate	M/50

The highest stimulating efficiency was that found for Na_3 citrate. On the basis of these and other experiments Mathews put forward the hypothesis that only the anions have a stimulating effect, while the cations have a depressing effect. The stimulating effect, he assumes, runs parallel to the solution tension of the anion. This theory meets with serious difficulties, since, first, it leaves unexplained why Ba salts have a higher stimulating effect than Na salts, and, second, why, as Loeb found, the stimulating effect of NaCl upon muscle is increased when some acid is added. Moreover, it is strange that while in NaCl the stimulation of the nerve may have a latent period of several hours, in the case of citrates the latent period may be only a few minutes. This time element indicates that another factor must enter into these results, and we shall see that this is probably the rapidity of diffusion of the salt into nerve or muscle. In 1899 Loeb had explained the accelerating action of acid upon the stimulating effect of NaCl upon muscle in this way. We have made a series of experiments which are not compatible with Mathews' theory, but which support the diffusion theory. According to this theory, the stimulating action of sodium salts upon nerve and muscle is inhibited by Ca and Mg, not on account of the depressing effect of the latter, as Mathews assumes, but because the Ca and Mg prevent the diffusion of salt into these organs.

II.

NH_4Cl is not able to stimulate nerve or muscle. The citrate of this base may have some stimulating action and this fact would

agree with Mathews' theory that the stimulating action depends only on the anion. We have found, however, that if in the molecule NH_4Cl the four hydrogen atoms are replaced by the ethyl group the resulting salt [tetraethylammonium chloride, $N(C_2H_5)_4Cl$] has a much higher stimulating effect upon the motor nerve than any sodium salt, even sodium citrate. As to our method, it may suffice to state that only the nerve was immersed in the solution, and the muscle was carefully protected from contact with the solution. The nerve muscle preparations (sciatic nerve and gastrocnemius muscle) from *Rana pipiens* were used. The solution was always made isotonic with an M/8 NaCl solution by the addition of the proper amount of cane sugar. The experiments were carried out in moist chambers and the exposed part of the nerve was carefully protected against drying out through frequent wetting with Ringer solution. The muscles were weighted. We used Merck's tetraethylammonium chloride,[4] while the other salts were Kahlbaum's.

Table III gives a comparison between the stimulating efficiency of corresponding salts of $N(C_2H_5)_4$ and of Na. Our values for the sodium salts do not entirely agree with those given by Mathews. We found that a M/12 NaCl solution is too weak to stimulate the nerve. In order to produce muscular contractions with certainty we had to immerse the nerve in M/4 NaCl. An M/8 NaCl solution was only in exceptional cases able to induce twitchings through the nerve. On the other hand, we found Na_3 citrate more efficient than in Mathews' experiments.

TABLE III.

Minimal stimulating concentrations of salts of Na and $N(C_2H_5)_4$.*

NaCl	>M/8	$N(C_2H_5)_4Cl$	about M/160
NaOH	M/20	$N(C_2H_5)_4OH$	" M/200
Na_3 citrate	M/100	$(N(C_2H_5)_4)_3$ citrate	" M/160

* In the hypotonic solution of these substances the solution was made isotonic with M/8 NaCl through the addition of cane sugar.

These data show clearly, first, that the stimulating effect of the tetraethylammonium salts is equal to or greater than that of

[4] In reality Merck's tetraethylammonium hydroxide was transformed into the chloride by adding HCl to the point of neutrality.

sodium citrate; and second, that the nature of the anion has little influence upon the efficiency of the tetraethylammonium salts. The powerful stimulating action of tetraethylammonium chloride is therefore due to the cation and not to the anion.

That it is the cation which determines the stimulating efficiency in this case is further proved by the fact that if the methyl group is substituted for the ethyl group the efficiency is lost. Tetramethylammonium chloride was ineffective in solutions isotonic with the nerve, as were also ammonium chloride, methylamine hydrochloride, ethylamine hydrochloride, diethylamine hydrochloride, and piperidine hydrochloride.

Attention should also be called to the fact that only strong bases act as nerve stimulants, while weak bases, such as NH_4OH and the amines, *e.g.*, diethylamine, triethylamine, butylamine, and benzylamine, are ineffective (except in hypertonic concentrations). This is almost the reverse of the relative effects of weak and strong bases in inducing artificial parthenogenesis.[5] It may be that the amines injure the nerve more than they do the egg.

III.

If the inhibitory effect of calcium salts upon the stimulating effect of sodium salts were due to an antagonism between anion and cation, as Mathews assumes, the amount of Ca required to suppress the stimulating action of a salt should depend chiefly upon the nature of the anion of the stimulating salt. If the stimulating action of tetraethylammonium chloride is due to the chloride, the amount of Ca required to suppress the stimulating action of this compound should be of the order of that required for the suppression of the stimulating action of NaCl. The contrary was found to be the case.

Solutions of sodium salts and of tetraethylammonium chloride were prepared so that with the proper addition of cane sugar the solution was always isotonic for the nerve. To 100 cc. of such a solution were added 1 or more cc. of M/8 $CaCl_2$ solution. The nerve was put into the solution and the number of cc. of M/8 $CaCl_2$ were observed that had to be added to 100 cc. of the stimulating solutions in order just to inhibit all the twitchings of the muscle when the nerve alone was immersed.

[5] Loeb, Artificial Parthenogenesis and Fertilization, Chicago, 1913.

TABLE IV.

Stimulating solution.	Cc. of M/8 $CaCl_2$ added to 100 cc. of stimulating solution.	
	Complete inhibition of twitchings.	No or incomplete inhibition of twitchings.
M/4 NaCl	2	1
M/8 Na_2SO_4	3.5	2, 2, 1, 1.5, 2.5, 3
M/64 Na_3 citrate	3, 5, 3, 5, 4	2, 3, 3.5
M/40 " "	7.5, 7.5	5, 6
M/32 " "	12.5, 12.5, 12.5, 12.5, 12.5	7.5, 10, 11, 10, 7.5
M/24 " "	20, 20, 22.5	7.5, 10, 12, 15, 17.5, 19, 20
M/16 " "	30	25, 20
M/64 Tetraethylammonium chloride	12.5	7.5, 10
M/40 Tetraethylammonium chloride	12.5	5, 7.5, 10
M/32 Tetraethylammonium chloride	12.5	7.5, 10, 12.5
M/24 Tetraethylammonium chloride	20	17.5, 15, 12, 10

There can be no doubt that the amount of $CaCl_2$ required for the suppression of twitchings in tetraethylammonium chloride is of a much higher order of magnitude than that required for the suppression of twitchings in sodium chloride. While the addition of 2 cc. of M/8 $CaCl_2$ suffices for this suppression in 100 cc. of M/4 NaCl, 12.5 cc. of M/8 $CaCl_2$ are required for the suppression of twitchings in a M/40 tetraethylammonium chloride solution, which is sixty times as much in the latter as in the former solution. This result is incompatible with the idea that the antagonism exists in both cases between the Cl^- and the Ca^{++} ion.

The amount of Ca required for the tetraethylammonium chloride solution is nearer the order of magnitude of that required for the sodium citrate solution.

Not only Ca but Mg also suppresses the twitchings of the nerve. The concentration of Mg required for complete inhibition of the stimulating effect of citrates and tetraethylammonium chloride is much greater than that of Ca, as Table V shows.

TABLE V.

Stimulating solution.	Cc. of M/8 $MgCl_2$ added to 100 cc. of stimulating solution.	
	Complete inhibition of twitchings.	No or incomplete inhibition of twitchings.
M/64 Na_3 citrate	10, 12	5, 6, 7.5, 8, 9
M/32 " "	25, 25, 30	15, 20, 20, 22.5
M/64 $N(C_2H_5)_4Cl$	40	20, 30
M/40 "	>40	40

It is obvious that the inhibiting power of Mg is less than that of Ca in this case. The same quantitative relation exists where Ca and Mg inhibit or retard the increase in permeability for sodium salts as in the experiments on *Fundulus*.[6]

IV.

It can be shown by a simple experiment that a "stimulating" action of $N(C_2H_5)_4Cl$ as well as of citrates is possible only if the nerve contains enough NaCl. The experiment consists in the following. A nerve is put for about $1\frac{1}{2}$ to 2 hours into an isotonic cane sugar solution until its electrical irritability (measured by the minimal distance of the two coils of an induction apparatus required to induce contractions of the muscle) is slightly diminished; *e.g.*, from 50 to 40 cm. distance. If then such a nerve is put into a M/64 solution of $N(C_2H_5)_4Cl$ in isotonic cane sugar, as a rule, no twitchings of the muscle will occur; if, however, NaCl is added to the solution containing the M/64 $N(C_2H_5)_4Cl$, twitchings may occur for a long time. Usually the experiment is made with the two sciatic nerves of the same frog which are put first into a pure sugar solution and afterwards in M/64 $N(C_2H_5)_4Cl$ solutions; the latter, in one case, being made isotonic through the addition of cane sugar, in the other, through the addition of NaCl.

The following may serve as an example. The two sciatic nerves were put into 7.6 per cent cane sugar solutions. At the beginning the faradic irritability measured by the distance in cm. of the two coils of the induction apparatus was at the distal end of the nerve 56 and 49. After 2 hours it was 33 and 35. One of

[6] Loeb, *J. Biol. Chem.*, 1914, xix, 431; *Biochem. Z.*, 1911, xxxii, 308.

the nerves (33 cm.) was put into M/64 $N(C_2H_5)_4Cl$ in M/8 NaCl, the other into M/64 $N(C_2H_5)_4Cl$ in 7.6 per cent cane sugar solution. In the NaCl the irritability rose in 15 minutes to 50 cm. and very soon the twitchings began. In the other the irritability remained the same, 35 cm., for an hour longer and then went down rapidly. No twitchings occurred. Other experiments confirmed this, showing that $N(C_2H_5)_4Cl$ can only stimulate a nerve if the latter contains enough NaCl, although its galvanic irritability may still be comparatively high.

The same can be shown for sodium citrate, which also stimulates the nerve only when the latter contains enough NaCl. The two sciatic nerves of the same frog were put for 4 hours and 20 minutes into a 7.6 per cent cane sugar solution. During this time the faradic irritability fell from 47 and 49 to 41 and 39 cm. coil distance. The former nerve was put into M/40 Na_3 citrate in 7.6 per cent cane sugar solution, the second nerve into M/40 Na_3 citrate in M/8 NaCl. In the latter the irritability rose in 35 minutes from 39 to 55 and then very vigorous twitchings began, which lasted for over an hour; *i.e.*, as long as the irritability remained high. Then it fell and the twitchings ceased. The nerve put into the citrate in sugar solution kept practically the same irritability (from 39 to 34) for the next 3 hours and no twitchings occurred. Both experiments, which were repeated, show clearly that the Na_3 citrate as well as the $N(C_2H_5)_4Cl$ can only stimulate the nerve when the latter has a sufficiently high irritability.

The same could be shown for sodium oxalate. When this salt is added to M/8 NaCl the twitchings last longer than if it is added to a sugar solution. Thus the twitchings lasted usually considerably longer when the nerve was immersed in 5 cc. M/10 sodium oxalate + 5 cc. M/8 NaCl than when it was immersed in 5 cc. M/10 sodium oxalate + 5 cc. 3/16 M cane sugar. In one case the twitchings lasted in the latter solution 20 minutes, in the former 38 minutes. After all the twitchings had ceased both nerves were transferred into M/8 NaCl solution. Now the twitchings began again and lasted for 80 minutes in both muscles. When, however, after the twitchings had ceased in the oxalate solution, the nerves were put into a pure cane sugar solution the twitchings did not begin again. The NaCl in both cases served the purpose of raising the irritability or keeping it at a high level.

v.

We now come to the discussion of a crucial question for the problem of chemical stimulation; namely, does the Ca inhibit the stimulating effect of $N(C_2H_5)_4Cl$ or of Na_3 citrate, on account of a "depressing" action, *i.e.*, by lowering the irritability of the nerve or merely by preventing these salts from diffusing sufficiently rapidly through the sheath of Schwann to the medullary sheaths or the axis cylinders inside the sheath? It has been proved sufficiently by experiments on *Fundulus* that, in this latter case at least, the antagonistic effect of $CaCl_2$ upon NaCl is due to the $CaCl_2$ preventing the diffusion of sodium salts through the membrane.[7]

It can first be shown that the Ca does not inhibit the efficiency of the stimulating salt by depressing the irritability of the nerve. The original threshold of two nerves of the same frog was 45 and 51. The one was put into M/24 Na_3 citrate in sugar, the other in 100 cc. M/24 Na_3 citrate + 20 cc. M/8 $CaCl_2$ in sugar (sufficient to make the solution isotonic). In the latter solution no twitchings occurred (in $3\frac{1}{2}$ hours). The threshold rose to 57 in 40 minutes and was still 49 after $3\frac{1}{2}$ hours. In the solution without Ca violent contractions began in 5 minutes when the threshold had risen to 58, and lasted 40 minutes, when the threshold was 54. In an hour the nerve was no longer irritable. It is natural to reason that the Ca prevented the citrate from diffusing into the nerve and the latter remained normal; while the nerve in the solution free from Ca was stimulated because the citrate diffused into the nerve which was killed on account of such diffusion. When the nerve, that had not twitched for 3 hours in the citrate solution containing Ca, was put into the citrate solution without Ca, twitchings began in 33 minutes and lasted for 20 minutes. During the time of twitching the irritability had risen from 49 to 60, due probably to the diffusion of the Na_3 citrate into the nerve. This was confirmed in a large number of experiments. No precipitate occurred in the concentration in which citrate and Ca were used.

The same was observed in the experiments with tetraethylam-

[7] Loeb, *Arch. ges. Physiol.*, 1905, cvii, 252; *Science*, 1912, xxxvi, 637; *Biochem. Z.*, 1912, xlvii, 127.

monium chloride. The addition of enough Ca to prevent the twitchings did not lower the threshold of faradic irritability. This eliminates the idea of Mathews that the inhibiting effect of Ca is due to a depression of the irritability of the nerve.

There is direct evidence, however, that the addition of Ca retards the rapidity of diffusion of the stimulating salts into the nerve. *If we add a considerable quantity of $CaCl_2$ to a stimulating salt solution, but not quite enough to inhibit the stimulating effect, we find that the latent period of stimulation is considerably lengthened.* The results on two nerves of the same frog were always compared (Table VI).

TABLE VI.

Latent Period for Stimulation of the Nerve in Tetraethylammonium Chloride.

Without calcium.		With calcium.	
	min.	*cc.*	*min.*
M/64 $N(C_2H_5)_4Cl$	6	+ 5.0 M/8 Ca	16
	2	+ 7.5 "	70
	5	+10.0 "	35
M/32 "	1	+ 7.5 "	11
	2	+10.0 "	35
	4	+12.5 "	48
M/24 "	3	+17.5 "	78
	5	+15.0 "	21

The addition of Ca to Na_3 citrate acts in a similar way. When enough Ca is added the latent period of stimulation of the nerve by Na_3 citrate is increased. Thus in M/24 Na_3 citrate the latent period was in five experiments between 5 and 8 minutes; in 100 cc. M/24 Na_3 citrate + 12.5, 15.0, 17.4, and 19 cc. M/8 $CaCl_2$, the latent periods were 23, 33, 40, and 34 minutes. Needless to say no precipitation of Ca citrate occurred in these mixtures.

It is, therefore, certain that Ca does not inhibit by a depression of the irritability of the nerve, but may inhibit by a diminution of the rate of diffusion of the stimulating salts into the nerve.

In addition it was found that when tetraethylammonium chloride solutions of different concentrations were tried, the latent period of stimulation for the nerve diminished with increasing concentration. Thus in M/32 $N(C_2H_5)_4Cl$ the latent period averaged 2

minutes, while in M/64, 6, 2, 5, 6, 10, 3, and 14 minutes were observed. When too little Ca was added the increase in the latent period was often not noticeable.

VI.

If we consider the idea that the Ca inhibits the chemical stimulation of the nerve by inhibiting the diffusion of the stimulating salts through the sheath of Schwann (or other envelopes) we must ask the question whether we cannot find an explanation for the great stimulating effect of certain salts like the citrates and those of $N(C_2H_5)_4$ on a similar basis. It can be pointed out that the stimulating action of the salts as shown in Tables I and II indicates that those salts which precipitate Ca contained at the surface of cells have also a greater stimulating power. This would concern the oxalates and fluorides, but not the citrates, or $N(C_2H_5)_4Cl$. The question may arise whether after all these latter salts which are powerful nerve or muscle stimulants without precipitating Ca are not those which possess a comparatively high degree of solubility in organic substances such as lipoids of the nerve or the envelopes of the muscles.

If it could be shown that the degree of solubility and permeability decides the stimulating value of these salts, we can understand why both cations as well as anions stimulate the nerve, and why the cation $N(C_2H_5)_4$ and the citrate anion should both have a high stimulating value. The diffusion of citrate into nerve and muscle is probably enhanced by the alkaline reaction of this solution.

VII.

This would still leave it unexplained why oxalates, citrates, and $N(C_2H_5)_4$ cannot stimulate the nerve after its irritability has been diminished by a longer treatment with a sugar solution; and why the addition of NaCl is required to make these salts efficient. As we have stated above, the NaCl is needed to bring the irritability of the nerve to a high value again. This proves that the nerve stimulants are only able to stimulate a highly irritable nerve; and that even a slight lowering of the electric irritability of the nerve renders these salts ineffective; although the nerve may still respond vigorously to galvanic currents of sufficient intensity.

The writer wonders whether the phenomena of chemical stimulation may not after all be cases of electrical stimulation which find their explanation in the electromotive phenomena described by Loeb and Beutner.[8] These authors observed that if we lead off from two spots of the surface of an intact animal or plant organ with two solutions of the same nature but of different concentration we notice the existence of considerable potential differences and these differences of potential follow Nernst's logarithmic formula. Such differences of potential originate also when we lead off from two substances of equal concentration but of different fat solubility. These E.M.F. originate at the boundary of watery and water-immiscible phases, and Beutner has developed their theory.[9]

When we immerse a nerve in a solution of Na_3 citrate or $N(C_2H_5)_4Cl$ these substances will probably not diffuse with equal rate through every spot of the sheath of Schwann. It will thus happen that at one spot at the surface of the medullary sheath of an axis cylinder a certain amount of $N(C_2H_5)_4Cl$ solution will collect, while a neighboring spot of the same axis cylinder contains this solution in a much lower concentration or not at all. This will necessitate a potential difference which will give rise to a minute current. If the irritability of the axis cylinder is high enough this will give rise to a twitching in the corresponding muscle fibers. It agrees with this view that the twitchings of the muscle due to chemical stimulation of its nerve are extremely irregular, which would correspond to the possible irregularities in the diffusion of these substances between the nerve fibrils. On this assumption we can also understand why only electrolytes can cause a chemical stimulation. The currents observed by Loeb and Beutner have nothing to do with the so called current of injury since they occur in perfectly normal surfaces, but are of a sufficiently high order of magnitude to stimulate nerve or muscle.

If the idea is correct that chemical stimulation is due to potential differences set up through an unequal rate of diffusion of the

[8] Loeb, J., and Beutner, R., *Biochem. Z.*, 1912, xli, 1; 1913, li, 288; 1914, lix, 195; *Science*, 1911, xxxiv, 884; 1913, xxxvii, 672. Loeb, *Science*, 1915, xlii, 643.

[9] Beutner, *Z. physik. Chem.*, 1914, lxxxvii, 385; *J. Am. Chem. Soc.*, 1914, xxxvi, 2040, 2045.

"stimulating" salt through the sheath of Schwann, it follows that these microscopic currents must exist inside of the sheath of Schwann at the boundary of individual axis cylinders or their medullary sheaths. Currents arising at the outer surface of the sheath of Schwann would find in the outside salt solution a conductor of electricity so much better than the nerve fiber that practically no current could go through the latter and hence no "stimulation" could occur.

If these hypothetical statements should be correct they would apply with equal force to the explanation of the chemical stimulation of muscle. In the chemical stimulation of muscle Loeb has formerly described a curious phenomenon which is observed only at the beginning of the experiment when the muscle is put into solutions of Na_3 citrate, Na_2H phosphate, Na_2 oxalate, and others. It consists in this, that the muscle which has not yet commenced to twitch in these solutions will go into powerful contractions whenever it is taken out of this solution and put into air, oil, or some other non-conductor.[10] This phenomenon would find its explanation in the assumption that in the beginning of the experiment the outer envelope of the muscle becomes unequally saturated or adsorbed by the stimulating salt and thus gives rise to local potential differences. As long as the muscle is in the solution the currents resulting from these potential differences will flow through the outside solution and no twitching occurs. If the muscle is taken out of the solution the currents are forced to flow through the adjacent muscle fibers and hence cause twitching. This phenomenon was inexplicable before the experiments of Loeb and Beutner were made.

While this explanation of chemical stimulation may seem satisfactory, it must not be forgotten that it is hypothetical. It would not, however, contradict the idea that changes in concentration of ions at the surface of the nerve fibril are the cause of *electrical* stimulation as Nernst's theory assumes; it would only mean that in the so called chemical stimulation of the nerve potential differences due to unequal diffusion in different spots in the nerve may lead to galvanic currents in the nerve.

An alternative explanation would be that the chemical stimu-

[10] Loeb, *Am. J. Physiol.*, 1900–01, iv, 423.

lation of Na_3 citrates, etc., or $N(C_2H_5)_4Cl$ upon nerve (or muscle) is due to some chemical or molecular influence of these salts upon the nerve or muscle fiber, provided the latter have a sufficiently high degree of irritability. This latter theory would make it difficult to understand why compounds of such different chemical constitution as $N(C_2H_5)_4Cl$ and Na_3 citrate could both stimulate, while a purely electrical theory would find no difficulty in such facts. Lillie has developed a theory whereby the increase in permeability itself is the essential feature in stimulation;[11] which would agree with some of the facts given in this paper but which would leave unexplained the main fact; namely, why only conductors of electricity will cause twitchings of nerve and muscle.

SUMMARY OF RESULTS.

1. It is shown that a non-stimulating salt like NH_4Cl can be made a powerful nerve stimulant if for the four hydrogen atoms ethyl groups are substituted. $N(C_2H_5)_4Cl$ is a more powerful stimulant for the nerve than sodium citrate.

2. There is little difference between the stimulating power of the chloride, hydroxide, and citrate of $N(C_2H_5)_4$, showing that in this case the cation and not the anion determines the high stimulating power.

3. This conclusion is supported by the fact that in order to inhibit the stimulating action of the cation $N(C_2H_5)_4$ through the addition of $CaCl_2$ the same high concentration of this latter salt is required as for the inhibition of the stimulating action of sodium citrate.

4. Both $N(C_2H_5)_4Cl$ and Na_3 citrate can only stimulate the nerve when the electrical irritability of the latter is at its normal height. If this irritability is only moderately diminished (by putting the nerve for 2 hours into an isotonic sugar solution) neither sodium citrate nor oxalate nor $N(C_2H_5)_4Cl$ will stimulate the nerve. If, however, the original irritability of the nerve is restored, by bathing the latter in NaCl, sodium citrate as well as $N(C_2H_5)_4Cl$ will stimulate the nerve again.

5. It can be shown that when enough Ca is added to a solution of $N(C_2H_5)_4Cl$ or Na_3 citrate to inhibit the stimulation, the irritability of the nerve is not lowered.

[11] Lillie, R. S., *Am. J. Physiol.*, 1911, xxviii, 197.

6. It can be shown that by adding a quantity of Ca not quite high enough to inhibit entirely the effect of the stimulating salts the latent period of stimulation is considerably increased, a fact which harmonizes with the assumption that the inhibiting effect of Ca is due to a prevention or retardation of the diffusion of the stimulating salt into the nerve.

7. All these facts contradict the theory of Mathews that only the anions have a stimulating effect and that Ca (and all the cations) have a depressing effect.

8. Possible explanations of the connection of diffusibility and stimulating action of salts are discussed.

THE UTILIZATION OF INOSITE IN THE DOG.*

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INTRODUCTION.

In connection with the investigations concerning the occurrence and chemical properties of phytin and inosite phosphoric acids[1] and the physiological rôle of these substances, we have undertaken a few experiments to determine the fate of inosite and its utilization in the animal organism. The previous work on phytin in this laboratory[2] concerned itself chiefly with the physiological relation of the phytin phosphorus to the metabolism. In the present paper we deal with the organic radical, inosite, of the phytin molecule.

The wide distribution of inosite in the vegetable and animal kingdoms has led to many investigations concerning the physiological function of this substance in the animal organism. Since inosite was first discovered in muscle by Scherer[3] it has been found in practically all the tissues and fluids of the body[4] and it

* Read before the Section of Biological Chemistry at the Urbana meeting of the American Chemical Society, April 20, 1916. The experimental work herein reported was carried out in the Institute of Physiology, University College, London.

[1] Anderson, R. J., *J. Biol. Chem.*, 1912, xi, 471; xii, 97, 447; 1912–13, xiii, 311; 1914, xvii, 141, 151, 165, 171; xviii, 425, 441; 1915, xx, 463, 475, 483, 493; *New York Agric. Exp. Station, Technical Bull.*, 19, 21, 22, 25, 1912; 32, 36, 1914; 40, 1915.

[2] Jordan, W. H., Hart, E. B., and Patten, A. J., *Am. J. Physiol.*, 1906, xvi, 268; *New York Agric. Exp. Station, Technical Bull.*, 1, 1906. Rose, A. R., *New York Agric. Exp. Station, Technical Bull.*, 20, 1912.

[3] Scherer, *Ann. Chem.*, 1850, lxxiii, 322.

[4] A review of the literature will be found in Jacobson, P., and Meyer, V., *Lehrbuch organ. Chem.*, 1902, ii, pt. i, 807.

appears to be a normal constituent of the urine.[5] In the vegetable kingdom, inosite is also widely distributed, occurring free in many plants and, particularly, conjugated with phosphoric acid as phytin or inosite hexaphosphoric acid[6] in seeds, roots, or tubers.

The earlier investigators[7] sought to establish some relation of inosite to the carbohydrates and to the elimination of sugar in diabetes. Külz[8] in an extensive series of experiments examined both normal and diabetic subjects. He found normal human urine practically free from inosite, but from the urine of six normal males after excessive drinking of beer or wine he obtained from 0.4 to 0.9 gm. of inosite. After feeding from 30 to 50 gm. of inosite to normal individuals he was able to recover only from 0.2 to 0.5 gm. of inosite from the urine. In the case of diabetics the same quantity of inosite gave practically the same result and there was no increase in the elimination of sugar. He thought for this reason that inosite might be utilized in the diabetic organism as a source of energy in place of sugar. By experiments on rabbits he also showed that inosite did not give rise to the formation of glycogen.

These studies were later continued by Mayer.[9] His results confirm those reported by Külz concerning the non-formation of glycogen from inosite. After feeding from 2 to 15 gm. of inosite *per os* to rabbits, only small amounts, from 2 to 2.4 per cent of the substance, were recovered from the urine. When it was given subcutaneously, however, he found that from 26 to 51 per cent of the inosite was eliminated unchanged in the urine. In addition to the inosite small quantities of lactic acid[10] were sometimes isolated from the urine after the inosite had been given subcutaneously.

[5] Hoppe-Seyler's Handb. Phys.- u. Path.-Chem. Analyse, Berlin, 7th edition, 1903, 220. Starkenstein, E., *Z. exp. Path. u. Therap.*, 1908–09, v, 378.

[6] A review of literature is given by Rose, A. R., *Biochem. Bull.*, 1912–13, ii, 21.

[7] See Starkenstein,[5] for a review of the literature.

[8] Külz, E., Sitzungsberichte Ges. z. Beförd. ges. Naturwissen., Marburg, 1876; Beitr. Path. u. Therap. Diabetes Mellitus, 1874, i.

[9] Mayer, P., *Biochem. Z.*, 1907, ii, 393.

[10] Mayer, *Biochem. Z.*, 1908, ix, 533.

Results very similar to the above were reported about the same time by Starkenstein.[5] About 5 per cent of the inosite given *per os* to rabbits was recovered in the urine. When the substance was injected subcutaneously about 42 per cent and after intravenous injections about 50 per cent of the inosite was eliminated in the urine. This author concludes that inosite is a normal cell constituent; that it is only slightly oxidized in the body because a large percentage of it is eliminated unchanged in the urine after being given in subcutaneous or intravenous injections, and that disappearance of inosite given *per os* is not permissible as proof of its being oxidized by the body since it may be destroyed by bacteria in the intestine.

It is evident from the work which has been done on this subject that inosite given *per os* is either largely destroyed by bacteria in the intestine or else it is stored or oxidized in the body, because very small amounts of it are eliminated in the urine. In the hope of throwing further light upon the fate of inosite in the animal organism we have carried out some respiration experiments with a dog which, in a fasting condition, was fed inosite *per os* just previous to being placed in the respiration apparatus. If inosite were oxidized in the body in the same manner as the carbohydrates or dextrose, for instance, then a rise in the respiratory quotient should be observed. The experiments have shown, however, that there was no appreciable rise in the respiratory quotient after giving 10 gm. of inosite. It is evident, therefore, that inosite is not oxidized in the dog in the manner of dextrose, and if oxidation or other changes take place they do so slowly and in such a way as not to affect the respiratory quotient during the first 2 hours following its ingestion.

It was observed, about 2 or 3 hours after the inosite had been given, that considerable diarrhea set in. Unfortunately it was not possible to separate the urine and the liquid excreta; these were therefore examined together. In one case, however, the liquid feces were voided outside of the metabolism cage and the urine was collected quite clear. When this clear urine was examined it was found to contain but 0.4 gm. of inosite. This shows, as in the case of man reported by Külz[8] and rabbits reported by Mayer[9] and Starkenstein,[5] that but small amounts of inosite are eliminated through the kidneys of the dog. When

the urine was mixed with liquid feces, however, very large quantities of inosite were obtained, and in one case as much as 77 per cent of the amount given was recovered from the mixed excreta.

EXPERIMENTAL.

The inosite used in this work was prepared from crude phytin by hydrolyzing this substance with dilute sulfuric acid in an autoclave. It was carefully purified by repeated recrystallization from water. The snow-white product finally obtained was free from ash and melted at 220° (uncorrected).

A small bitch weighing 5,700 gm. was selected for these experiments and trained for use in the respiration apparatus. The animal was kept in a metabolism cage and fed once a day, in the evening, on cooked meat and dog biscuit, but during the period when inosite was given meat was the sole food.

The Benedict respiration apparatus described by Moorhouse, Patterson, and Stephenson[11] was used. The carbon dioxide and oxygen measurements were made as there described, and for particulars we refer to the above paper.

The animal was fed about 5 p.m., and the respiration observations were made about 18 hours afterwards. The quotients in this fasting condition are given in Table I. The figures obtained lie within the limits usually observed under these conditions.

TABLE I.

Fasting Respiratory Quotients.

Date.	Length of period.	CO_2 per minute.	O_2 per minute.	R. Q.	Remarks.
	min.	*cc.*	*cc.*		
Feb. 11	42	40.35	54.61	0.739	Usual movements.
" 12	64	44.62	58.65	0.760	Somewhat restless.
" 15	58	42.03	55.72	0.754	" "
" 24	46	35.95	45.65	0.787	Very quiet.

The inosite was given dissolved in about 70 cc. of warm water. This solution was taken at first just as readily as one of glucose,

[11] Moorhouse, V. H. K., Patterson, S. W., and Stephenson, M., *Biochem. J.*, 1915, ix, 176.

but after a while the inosite solution was absolutely refused and it had to be given by the stomach tube. A few minutes after the inosite had been given the animal was placed in the respiration apparatus, and the observation periods varied from $1\frac{1}{2}$ to about 2 hours. It will be noticed by referring to the figures in Table II that the respiratory quotients during these periods are slightly

TABLE II.

Respiratory Quotients after Giving 10 Gm. of Inosite.

Date.	Length of period.	CO_2 per minute.	O_2 per minute.	R. Q.	Remarks.
	min.	*cc.*	*cc.*		
Feb. 11	90	45.18	57.73	0.782	Restless.
" 12	126	44.72	56.50	0.791	"
" 15	123	44.13	56.50	0.780	"
" 17	111	43.38	58.08	0.747	"

higher than those in the fasting condition (Table I). The difference is very slight, however, and it is doubtful if the small rise in the respiratory quotient is due to oxidation of the inosite. The animal showed signs of discomfort during the inosite periods and was more or less restless, consequently the oxygen measurement was more difficult. Diarrhea usually set in a short time after the animal was removed from the respiration apparatus.

For comparison with the quotients tabulated above some figures after feeding glucose are given in Table III.

TABLE III.

Respiratory Quotients after Feeding Glucose.

Date.	Glucose.	Length of period.	CO_2 per minute.	O_2 per minute.	R. Q.	Remarks.
	gm.	*min.*	*cc.*	*cc.*		
Feb. 9	20	63	59.77	60.84	0.982	Restless.
" 18	10	78	65.20	66.02	0.989	"

In Table IV are given the amounts of inosite[12] recovered from the urine and liquid feces. In this connection it must be stated that only the liquid portion of the excreta which collected in the bottle underneath the metabolism cage was analyzed for inosite.

[12] The inosite was isolated by the method of P. Mayer.[9]

TABLE IV.

Inosite Recovered from Urine and Liquid Feces.

Experiment.	Inosite given.	Inosite recovered.		Remarks.
	gm.	*gm.*	*per cent*	
1	10	0.40	4.0	Urine clear.
2	10	4.00	40.0	" plus feces.
3	10	1.27	12.7	" " "
4	10	2.25	22.5	" " "
5	10	6.30	63.0	" " "
6	10	7.70	77.0	" " "
1st day	None.	0.20		" clear.
2nd "				
3rd "	"	Trace.		" "

The variation in the amounts of inosite recovered in the first four experiments is due no doubt to the fact that in these periods the dog was used for the respiration observations and shortly after being removed from the respiration apparatus diarrhea set in and these liquid stools were lost. During the fifth and sixth experiments, however, the dog was kept in the metabolism cage the whole time and all the liquid feces and the urine were collected together.

After the sixth experiment the clear urine was collected for the next 3 days, and analyzed for inosite. By referring to the table it will be noticed that the combined urine of the 1st and 2nd days contained only 0.2 gm. of inosite while on the 3rd day a mere trace was obtained. This shows that practically all the inosite given is eliminated within 24 hours; evidently, therefore, either none or very little of it can be stored in the body.

Since the clear urine (Table IV) contained little and the urine plus liquid feces contained much inosite, it would seem that by far the greater portion of the substance was not absorbed but that it was eliminated through the bowel. In order to determine to what extent inosite is absorbed in the intestine the following experiment was made. A section about 2 feet long of the small intestine of a dog was exposed[13] and washed out with physiological salt solution. One end was ligatured, a solution of 3 gm. of inosite in 50 cc. of warm water was introduced, and the other

[13] The operation was performed by Professor E. H. Starling.

end was also ligatured. The whole was replaced in the abdominal cavity, the incision loosely closed, and the animal kept under chloroform for 2 hours. On removing the section of the bowel it was quite distended and contained 90 cc. of liquid. From this liquid 2.95 gm. of inosite were isolated; *i.e.*, the substance was recovered practically quantitatively.

CONCLUSIONS.

Inosite is not utilized to any extent by the dog. It is not stored or oxidized in the body but the greater portion of it is excreted unchanged. When given at the rate of 2 gm. per kg. of body weight it does not cause a rise in the respiratory quotient.

As much as 77 per cent of the amount given may be recovered from the excreta.

Inosite is absorbed very slowly from the intestine, hence it causes a more or less severe diarrhea. As a result it is largely eliminated with the feces and only a small portion is excreted by the kidneys.

The author acknowledges his indebtedness to Professer E. H. Starling for help and advice in carrying out the above e periments, and to Dr. V. H. K. Moorhouse for assistance in using the respiration apparatus.

THE UTILIZATION OF INOSITE IN THE ANIMAL ORGANISM. THE EFFECT OF INOSITE UPON THE METABOLISM OF MAN.*

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(Received for publication, May 6, 1916.)

INTRODUCTION.

The objects of this investigation were to determine whether the ingestion of inosite has any noticeable influence upon the metabolism of man measurable in terms of the nitrogen and phosphorus excretion, and also the fate of inosite in the human body and its channels of elimination.

It has been shown by one of us[1] that inosite is not utilized to any extent by the dog, that it acts as a strong purgative, and that the greater portion is eliminated unchanged through the bowel.

That inosite causes more or less severe diarrhea in both man and animals when given in large quantities has been observed previously by Külz[2] who thought that the diarrhea was caused by the transformation of the inosite into lactic acid in the intestine. In normal or diabetic human subjects from 30 to 50 gm. of inosite per day caused considerable diarrhea and only from 0.2 to 0.5 gm. of the inosite was recovered from the urine. This author does not report the examination of the feces of his subjects for inosite. In the case of rabbits the above author found that inosite caused diarrhea and only small quantities of the substance were recovered from the urine. Results very similar to the above

* Read before the Section of Biological Chemistry at the Urbana meeting of the American Chemical Society, April 20, 1916.

[1] Anderson, R. J., *J. Biol. Chem.*, 1916, xxv, 391.

[2] Külz, E., Sitzungsberichte Ges. z. Beförd. ges. Naturwissen., Marburg, 1876.

have been reported by Mayer[3] and Starkenstein[4] who both used rabbits as subjects.

Of the previous investigations concerning the physiological rôle of inosite in man those of Külz[2] are probably the most thorough; he had an unusually large quantity of inosite at his disposal for experimental purposes, having prepared about a pound of it from green beans. The high price and the small available quantities of inosite have presented difficulties in carrying out investigations with this substance. For the present work we prepared several hundred grams of pure inosite as described on page 394.

Our results confirm those of previous investigators that inosite given *per os* disappears and only small quantities are eliminated in the urine. Külz[2] recovered only about 0.9 per cent of the inosite given from the urine of human subjects. Our figures, however, are about ten times greater; *i.e.*, we recovered from the urine nearly 9 per cent of the ingested inosite. Careful examination of the feces was made but we failed to obtain a trace of inosite. Consequently the urine is the only channel of elimination of inosite in man.

In what manner the balance, about 91 per cent, of the inosite is utilized or destroyed is uncertain. It has been shown by the investigations of Meillère[5] and also by Starkenstein,[4] that inosite is destroyed by the colon bacillus. It has also been shown by Hilger[6] and Vohl[7] that inosite is transformed into lactic acid by the bacteria found in putrid cheese. Experiments by Starkenstein[4] indicate that inosite is destroyed by autolytic ferments of muscles and the liver with the simultaneous increase of lactic acid.

Our results do not show whether the inosite was destroyed by bacteria in the intestine or underwent oxidation in the body. If the inosite was changed into lactic acid it is evident that only traces of it were excreted as such, because there was no appreciable increase of ammonia in the urine (Table III). At present we can only record the fact that of the inosite given only about 9 per cent escaped destruction and was recovered from the urine.

[3] Mayer, P., *Biochem. Z.*, 1907, ii, 393.
[4] Starkenstein, E., *Z. exp. Path. u. Therap.*, 1908–09, v, 378.
[5] Meillère, G., *Compt. rend. Soc. biol.*, 1907, lxii, 1096.
[6] Hilger, *Ann. Chem.*, 1871, clx, 333.
[7] Vohl, H., *Ber. chem. Ges.*, 1876, ix, 984.

It is evident from the data presented in the experimental part that, except for the increased excretion of creatinine in the after-period, the ingestion of inosite at the rate of about 0.5 gm. per kg. of body weight has no appreciable influence upon the metabolism of man.

EXPERIMENTAL.

To determine the channels of elimination of inosite in man we carried out the following preliminary experiment. One of us (B.) received 30 gm. of inosite dissolved in water in three equal portions during the day. The following morning a solution of 10 gm. of inosite was given. About 4 hours after the first 10 gm. of inosite were taken a very loose watery stool was passed and during the 24 hours there were three more very watery stools. The second day the stools were almost of normal consistency. During the first and second days the subject complained of an uneasy or uncomfortable feeling and of a peculiar sensation in the chest and abdominal muscles. This feeling disappeared towards the end of the 3rd day and on the 4th day he felt normal again.

The urine and feces were collected in 24 hour periods and examined for inosite. For the isolation of the inosite we used the method of Mayer[3] which is briefly as follows: The urine was evaporated to about one-fourth of its volume and precipitated with excess of lead acetate. After standing a short while the precipitate was filtered off on a Buchner funnel and washed with water. The filtrate was heated to boiling and precipitated with excess of basic lead acetate and finally rendered strongly alkaline with concentrated ammonia. After standing for 24 hours the precipitate was filtered on a Buchner funnel and washed with water. It was then suspended in water and decomposed with hydrogen sulfide. After removing the lead sulfide, the filtrate was evaporated on the water bath almost to dryness. The residue was taken up in a little hot water, decolorized with animal charcoal, again evaporated to small bulk, transferred to an Erlenmeyer flask, and brought to crystallization by the addition of about ten volumes of absolute alcohol. After the greater portion of the inosite had crystallized out ether was added until the solution turned cloudy and the whole was allowed to stand in

the ice box over night. The crystals were then filtered off, washed with alcohol and ether, dried in the air, and weighed.

The feces were examined as follows: The fresh material was stirred up with water until of uniform consistency. Lead acetate was then added and after standing for some time it was filtered and washed. The filtrate was evaporated, precipitated with basic lead acetate, etc., as mentioned above.

The amount of inosite recovered in the excreta is given in Table I.

TABLE I.

Day.	Inosite given.	Inosite recovered from the urine.		Inosite recovered from the feces.
	gm.	*gm.*	*per cent*	
1	30	2.6	8.66	None.
2	10	1.1	11.00	"
3	None.	Trace.		"

The results show that in man a small quantity of the ingested inosite is eliminated in the urine and that the feces contain none; also that it is either absorbed and oxidized in the body or else destroyed in the intestine, and that the excretion of unchanged inosite is complete in 24 hours, because on the 3rd day the urine contained only a trace of the substance. We find that the quantity of inosite excreted by the kidneys is about ten times greater than found by Külz. This is probably due to the improved method of isolating the substance.

In a second more extensive experiment we have sought to discover if the ingestion of inosite produced any change or disturbance in the metabolism. For this purpose we selected a period of 8 days during which the food of the subject (B.) was carefully regulated. Unfortunately we failed to maintain any control of the food intake during the days following the actual experiment or after-period. The results are vitiated also by the fact that the period over which our experiment extended was not of sufficient length to bring the subject to a condition of nitrogen equilibrium and that we analyzed only one sample of each article of food. Since it was a liberal and mixed diet consisting of meat, eggs, milk, potatoes, bread, etc., it is probable that the actual intake during the whole period was different from what we figured from the result of one analysis. Despite these drawbacks we feel justi-

fied in publishing briefly the results obtained because these results are negative; *i.e.*, our observations have failed to reveal any noteworthy or striking change in the general metabolism as a result of the ingestion of inosite.

During this second experiment which was begun a few months after the one reported above, we began the inosite period by giving only 15 gm. of the substance the 1st day. On the 2nd and subsequent days 30 gm. of inosite were given. In this way the uncomfortable diarrhea was avoided. The stools were more frequent and softer than usual, but towards the end of the period they were nearly of normal consistency. There were no feelings of discomfort, in fact the subject felt practically normal throughout the experiment, performing his laboratory work in the usual manner.

The total nitrogen and phosphorus balance, with the caution mentioned above, is given in Table II. The difficulty of bringing man to a nitrogen equilibrium and the fact that the diet gave a nitrogen intake quite a little below the usual average daily intake of the subject makes the minus nitrogen and phosphorus balance have no special significance.

TABLE II.

Total Nitrogen and Phosphorus Balance.

Day.	Preliminary period.				Inosite period.			
	1	2	3	4	5	6	7	8
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
Nitrogen intake....	15.254	15.483	11.781	12.131	12.880	11.355	11.151	11.249
Nitrogen excretion.	15.018	13.602	14.233	12.667	13.943	11.682	13.147	11.821
Phosphorus intake....	1.832	1.708	1.362	1.384	1.592	1.318	1.323	1.330
Phosphorus excretion.	2.1267	1.6626	1.8688	1.5634	1.8469	1.4029	1.5119	1.4680

		gm.		*gm.*
Total	nitrogen intake, preliminary period,	54.649;	inosite period,	46.635
"	" excretion, " "	55.520;	" "	50.593
	Minus nitrogen balance,	0.871;		3.958
"	phosphorus intake, preliminary period,	6.286;	inosite period,	5.563
"	" excretion, " "	7.2215;	" "	6.2297
	Minus phosphorus balance,	0.9355;	" "	0.6667

Figures covering the elimination of nitrogen in the urine are tabulated in Table III. There was a considerable rise in the creatinine output which began on the last day of the inosite period and extended through the whole of the after-period, the first 2 days of the after-period showing a marked increase in creatinine elimination. During the after-period the subject ate the same diet as during the rest of the experiment but the food was not accurately weighed. In view of this fact, the increased creatinine output during the after-period would seem to be an important observation and a matter worthy of further study.

The uric acid, creatinine, and ammonia were determined by the Folin methods.

TABLE III.

Analysis of the Urine. Nitrogen Elimination.

Preliminary Period.

Day	1	2	3	4
Volume of urine, cc.	1,420	1,480	1,170	710
Specific gravity	1.019	1.015	1.020	1.028
Total nitrogen, gm.	12.908	12.802	12.273	11.204
Uric acid, gm.	0.636	0.658	0.585	0.546
Creatinine, gm.	1.198	1.199	1.050	1.150
Ammonia nitrogen, gm.	0.965	0.864	0.959	0.899

Inosite Period.

Day	5	6	7	8
Volume of urine, cc.	770	810	1,450	900
Specific gravity	1.026	1.026	1.016	1.022
Total nitrogen, gm.	11.504	9.914	11.296	9.234
Uric acid, gm.	0.550	0.463	0.520	0.692
Creatinine, gm.	1.299	1.010	1.068	1.448
Ammonia nitrogen, gm.	1.089	0.868	1.125	0.918

After-Period.

Day	9	10	11	12
Volume of urine, cc.	1,175	1,390	1,550	1,070
Specific gravity	1.022	1.022	1.016	1.023
Total nitrogen, gm.	12.009	13.232	8.680	8.988
Uric acid, gm.	0.628	0.731	0.620	0.767
Creatinine, gm.	1.729	2.183	1.141	1.332
Ammonia nitrogen, gm.	0.940	0.970	0.639	0.683

The phosphorus elimination in the urine is recorded in Table IV. The total phosphorus was determined after destroying the organic matter by the Neumann method. The inorganic phosphorus is that quantity which was directly precipitated by ammonium molybdate after acidifying the urine with nitric acid and adding ammonium nitrate. The organic phosphorus was found by difference. It will be noticed by referring to the table that the organic phosphorus varied from 0 to 0.0178 gm. with an average of 0.0102 gm. per day. This agrees with the average quantity of organic phosphorus usually found in normal urine.[8]

TABLE IV.

Elimination of Phosphorus in the Urine.

Preliminary Period.

Day.	1		2		3		4	
Volume of urine, cc.	1,420		1,480		1,170		710	
	per cent	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*
Total phosphorus...	0.0835	1.1857	0.0843	1.2476	0.0946	1.1068	0.1433	1.0174
Inorganic phosphorus.....	0.0826	1.1729	0.0831	1.2298	0.0946	1.1068	0.1413	1.0032
Organic phosphorus.	0.0009	0.0128	0.0012	0.0178	0	0	0.0020	0.0142

Inosite Period.

Day.	5		6		7		8	
Volume of urine, cc.	770		810		1,450		900	
	per cent	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*	*per cent*	*gm.*
Total phosphorus...	0.1370	1.0549	0.1174	0.9509	0.0742	1.0759	0.1030	0.9270
Inorganic phosphorus.....	0.1358	1.0456	0.1165	0.9436	0.0738	1.0701	0.1014	0.9126
Organic phosphorus.	0.0012	0.0093	0.0009	0.0073	0.0004	0.0058	0.0016	0.0144

The amount of nitrogen and phosphorus excreted in the feces is given in Table V. A notable rise in the nitrogen excretion is observed during the inosite period. This was no doubt due to the more frequent stools and consequent less complete absorption in the intestine. Since it was shown in the preliminary experi-

[8] For a review of the literature see Forbes, E. B., and Keith, M. H., *Ohio Agric. Exp. Station, Technical Bull.*, 5, 1914, 190.

TABLE V.

Nitrogen and Phosphorus Excretion in the Feces.

Day.	Preliminary period.				Inosite period.			
	1	2	3	4	5	6	7	8
Nitrogen, gm.....	2.110	0.800	1.960	1.463	2.439	1.768	1.851	2.587
Phosphorus, gm.....	0.941	0.415	0.762	0.546	0.792	0.452	0.436	0.541

ment that inosite was eliminated only in the urine the feces were not examined this time for this substance.

In Table VI the daily intake of inosite and the quantities recovered from the urine are recorded. The same method of isolation was used as before. The inosite was excreted in about the same proportion as in the preliminary experiment. The total intake amounted to 105 gm. of inosite and only about 9 gm. were recovered. The balance, 96 gm., was either oxidized in the body or destroyed by bacteria in the intestine. Evidently the inosite could not have been stored in the body because the elimination ceases with the intake.

TABLE VI.

Inosite Intake and Excretion.

Day.	5	6	7	8	9	10	11	12
Volume of urine, cc....	770	810	1,450	900	1,175	1,390	1,550	1,070
Inosite intake, gm.....	15	30	30	30	None.	None.	None.	None.
Inosite excretion, gm....	0.38	2.5	3.19	2.5	0.47	Trace.	Trace.	Trace.

Total intake, 105 gm. of inosite.
Inosite recovered, 9.04 gm. or 8.6 per cent.

SUMMARY.

When inosite is taken at the rate of about 0.5 gm. per kg. of body weight per day it produces some diarrhea at first or frequent soft stools. After a few days the stools, although more frequent than usual, are nearly of normal consistency.

Except for the increased excretion of creatinine in the after-period, for which we can now offer no explanation, we find that the ingestion of inosite has no marked or appreciable effect upon the general metabolism of man.

About 9 per cent of the inosite taken *per os* is eliminated unchanged in the urine, but none in the feces. In what manner the balance, or about 91 per cent, of the inosite is utilized we have not been able to determine.

THE NATURE OF THE DISEASE DUE TO THE EXCLUSIVE DIET OF OATS IN GUINEA PIGS AND RABBITS.

By CASIMIR FUNK.

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(Received for publication, May 9, 1916.)

The subject of the present paper was suggested by the investigations of Morgen and Beger,[1] who fed rabbits on a diet composed wholly of oats. Their results coincided with those obtained by other authors,[2] who also were unable to keep these animals alive for more than a few months. To overcome this they added certain inorganic salts to the diet. Sodium chloride was found to stimulate the food intake, and calcium carbonate to prevent loss in weight, but these failed to decrease the mortality. They concluded that the disease which developed as the result of an exclusive diet of oats was due to acidosis, and tried to prove this by experiments with sodium bicarbonate. When this salt was added to the oats the animals remained in apparently good condition and with one exception lived for more than half a year, when the experiment was discontinued.

Experiments similar to those described by Morgen and Beger have previously been reported. Lunin[3] fed mice on a mixture of casein, fat, and sugar, and found that when sodium carbonate was added to neutralize the sulfuric acid formed from protein the mice lived longer. Weiser[4] fed growing pigs on corn, and the chemical analysis of urine and feces showed a positive nitrogen, but a negative phosphorus and calcium balance. When calcium carbonate was added the latter balances became positive. As opposed to this, Schaumann[5] has found that rabbits are unable to live on corn even upon the addition of calcium carbonate, but do well when dried yeast (5 per cent) is added. Laqueur[6] has shown the beneficial effect of sodium chloride in starving rabbits.

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[1] Morgen, A., and Beger, C., *Z. physiol. Chem.*, 1915, xciv, 324.

[2] Holst, A., and Frölich, T., *J. Hyg.*, 1907, vii, 634.

[3] Lunin, N., *Z. physiol. Chem.*, 1881, v, 31.

[4] Weiser, S., *Biochem. Z.*, 1912, xliv, 279.

[5] Schaumann, H., *Arch. Schiffs- u. Tropenhyg.*, 1914, xviii, 363.

[6] Laqueur, E., Résumé des comm. et demonstr., *IX Congr. Physiol.*, Groningen, 1913.

The question arises whether the change that occurs in guinea pigs and rabbits fed on a diet of oats can be considered as scurvy, notwithstanding its similarity to human scurvy. Judging by the inactivity of the known antiscorbutics in the disease described, I have already[7] expressed some doubt as to its nature, as the symptoms of starvation greatly complicate the diagnosis.

The following investigations were undertaken to study the effect of feeding oats to rabbits, guinea pigs, and also to rats, with special reference to the effect of the addition of sodium bicarbonate and the action of antiscorbutics. The conclusions were in accordance with those of Morgen and Beger that the addition of sodium bicarbonate when given in small quantities has a marked beneficial effect in rabbits. This result, together with the inactivity of a vitamine addition in the form of dried yeast, and the failure of the known antiscorbutics, indicates that this condition is probably due to acidosis. In guinea pigs the result was different, as the sodium bicarbonate was found to be without effect and the addition of antiscorbutics a marked failure, making the diagnosis of scurvy uncertain. It was also found that the condition described as scurvy in animals is not suitable for testing chemical fractions obtained from the antiscorbutics, but we do not wish to generalize this statement as we have had no experience with scurvy in monkeys (Hart and Lessing[8]). Whether scurvy in rats will prove a success in this respect is at present under investigation in this laboratory.

The beneficial action of sodium bicarbonate in rabbits fed on oats does not indicate that scurvy is a deficiency disease, since when fed to pigeons, oats are found to contain a sufficient amount of beri-beri vitamine to prevent the outbreak of beri-beri. It even seems possible that rabbits are able to prepare the scurvy vitamine from the beri-beri vitamine present in oats when sodium bicarbonate is supplied.

In the original experiments of Morgen and Beger rabbits were fed oats with sodium bicarbonate added to the extent of 1 per cent, and occasionally other salts were given. In my first experiment the sodium bicarbonate (1 gm.) was introduced by means

[7] Funk, C., Die Vitamine, Wiesbaden, 1914.

[8] Hart, C., and Lessing, O., Der Skorbut der kleinen Kinder, Stuttgart, 1913.

CORRECTION.

On page 410, Vol. xxv, No. 3, July, 1916, the twenty-seventh line of text should read *is not an argument against scurvy being a deficiency disease.*

of a stomach tube; in another series of experiments an equivalent amount of hydrochloric acid was given ½ hour after the bicarbonate in order to test whether the presence of sodium bicarbonate would not be a sufficient stimulation. Care was taken that the quantity of liquid given was identical in both cases. The result of this experiment is given in Table I, and although sodium bicarbonate stimulated the appetite, it had no life-prolonging action. A possible reason for this might be that the salt was given in too large quantity, 1 gm. at a time having been administered. The food was weighed back every day, but averages will be given for 3 weeks only. The dead animals showed marked scorbutic changes.

TABLE I.

		Weight.		Food intake.		Death after
		Initial.	Final.	Initial.	Final.	
		gm.	*gm.*	*gm.*	*gm.*	*days*
Controls on oats..	I	1,155	775	35	23	49
	II	1,566	880	29	19	70
	III	1,305	884	40	22	57
	IV	1,311	1,035	44	28	28
Oats and $NaHCO_3$	I	1,480	1,232	65	33	64
	II	1,387	1,112	50	40	37
	III	1,140	998	41	28	49
	IV	1,276	1,592	65	54	52
Oats, $NaHCO_3$, and HCl.....	I	1,623	1,194	53	26	58
	II	1,380	1,340	38	38	27
	III	1,470	1,082	40	36	35
	IV	1,535	1,445	59	59	36

In the next experiment the sodium bicarbonate was added to the extent of 1 per cent of the diet. The method again differed slightly from that used by Morgen and Beger, as the salt was given continuously instead of in periods, and this possibly accounts for the result obtained. It was found that this salt increased the appetite, but two out of four animals died showing scorbutic changes (Nos. II and III). The first rabbit was killed after 119 days and carefully examined, but no pathological changes were found except a slight fragility of the bones and an enlarged right heart.

TABLE II.

		Weight.		Food intake.		Death after
		Initial.	Final.	Initial.	Final.	
		gm.	*gm.*	*gm.*	*gm.*	*days*
Oats and $NaHCO_3$ . . .	I	1,540	1,142	76	29	119
	II	1,620	1,088	67	22	107
	III	1,383	867	55	19	100
	IV	1,730	1,493	66	36	Alive after 123 days.

The final food intake was found to be much smaller than the initial one, indicating perhaps that all animals would eventually die. This again does not corroborate Morgen and Beger's results. That rabbits show great variability when kept on oats is demonstrated in Table III. In this experiment two rabbits were kept on oats alone much longer than the controls of the first experiment. The first of these rabbits was pregnant and gave birth to eight apparently normal young after 40 days on a diet of oats. This animal lived during 95 days on this diet and was in good condition when the experiment was discontinued, showing only a moderate loss in weight.

TABLE III.

		Weight.		Duration.
		Initial.	Final.	
		gm.	*gm.*	*days*
Oats.	I (Pregnant.)	3,690	3,309	95
	II	2,036	1,500	76

The Action of Yeast and Various Antiscorbutics in Rabbits Fed on Oats.

In this experiment four rabbits were given oats with 1 per cent yeast for 12 days, then 10 to 20 cc. of potato juice for 6 days, and finally yeast and potato juice together. The juice was prepared by grinding fresh potatoes and filtering the pulp with suction without diluting it. The figures under "Food intake" are successive weekly averages. The experiment, however, was discontinued as the food intake was poor in spite of the additions.

TABLE IV.

		Weight.		Average food intake.	Duration.	Result.
		Initial.	Final.			
		gm.	*gm.*	*gm.*	*days*	
Oats, yeast, and potato juice.	I	1,470	1,254	62, 52, 26, 12	35	Died.
	II	1,260	1,143	63, 59, 26, 8	35	Discontinued.
	III	1,258	1,024	47, 34, 21, 14	35	Died.
	IV	1,265	1,342	81, 62, 25, 35	35	Discontinued.

Rabbits I and III showed scorbutic changes at autopsy. The experiment with yeast was repeated on several other rabbits with identical results.

Besides yeast and potato juice a series of experiments were undertaken with germinated oats, which, according to Fürst[9] prevent the outbreak of scurvy in guinea pigs. Apparently the soaked seeds were not a suitable diet, as the animals died in a short time.

Experiments on Guinea Pigs.

The experiment in which sodium bicarbonate was given to rabbits was repeated with guinea pigs, the salt being mixed with

TABLE V.

		Weight.		Food intake.		Death from scurvy after
		Initial.	Final.	Initial.	Final.	
		gm.	*gm.*			*days*
Oats.	I	388	239	Food intake steadily decreasing.		30
	II	366	274			24
	III	371	252			22
	IV	392	305			22
				1st week.	2nd week.	
				gm.	*gm.*	
Oats and $NaHCO_3$.	I	502	364	19	2	18
	II	539	383	25	24	24
	III	644	468	29	2	17
	IV	525	543	41	22	17

[9] Fürst, V., *Z. Hyg. u. Infectionskrankh.*, 1912, lxxii, 121.

the oats. The result obtained differed from that with rabbits, in that the animals died earlier than the controls. We therefore are not justified in considering that the pathological condition due to the feeding of oats is acidosis.

In Table VI the results of the experiments with various antiscorbutics on guinea pigs are summarized. Experiments were undertaken with potato juice, milk, lime juice, and with various fractions prepared according to a previous paper.[10] The best preventive action was obtained with milk, but only when large quantities were given.

TABLE VI.

		Weight.		Duration.	Result.
		Initial.	Final.		
		gm.	*gm.*	*days*	
Controls on oats.	I	425	315	26	Died; symptoms of scurvy.
	II	475	330	24	" " " "
	III	615	380	16	" " " "
	IV	457	250	27	" " " "
	V	427	250	22	" " " "
	VI	390	230	21	" " " "
	VII	348	207	30	" " " "
	VIII	372	231	24	" " " "
	IX	267	154	27	
	X	261	164	25	
	XI	263	177	22	
	XII	248	190	24	Died; symptoms of scurvy.
	XIII	310	180	24	" " " "

In the last period nothing or very little was eaten. Average duration 24 days. Average loss in weight 37 per cent.

[10] Funk, *Biochem. J.*, 1913, vii, 81.

TABLE VI—*Concluded.*

		Weight.		Duration.	Result.
		Initial.	Final.		
Phosphotungstic acid fraction from 15 liters of lime juice made up to 1 liter. 20 cc. daily, and oats.	I	484	325	24	Died of scurvy.
		640	345	28	" " "
		752	600	20	" " " Average duration 25 days.
		542	290	30	Died of scurvy. Average loss in weight 34 per cent.
Phosphotungstic filtrate fraction in amount corresponding to the above, and oats.		880	700	14	Alive.
		700	425	12	Died of scurvy.
		605	470	16	" " "
		435	335	18	" " "
25 cc. potato juice daily and oats.		392	238	34	" " " Loss in weight 38 per cent.
		391	244	36	Died of scurvy.
25 cc. milk daily and oats.		467	292	38	" " "
		447	393	50	Alive.
50 cc. milk daily and oats.		395	375	50	"
		354	440	50	"
Protein-free milk, corresponding to 200 cc. fresh milk, and oats.		438	269	29	Died of scurvy.
		319	185	38	" " "
		342	247	33	" " "
		415	287	35	" " "
Alcoholic extract from 3 liters of milk made to 300 cc., 40 cc. daily, and oats.		449	268	24	" " "
		340	282	14	" " "
30 gm. milk powder extracted with alcohol added, and oats.		163	90	25	" " "
		201	161	92	Alive.
The same diet as above but no extract.		142	168	92	"
		112	90	24	Died of scurvy.

Rats on Diet of Oats.

Rats can be maintained on oats and white bread for a long time, although it was found that young rats do not grow on this diet and the addition of sodium bicarbonate had no beneficial action. The failure of young rats to grow on oats coincides with experiments[11] on young chickens that failed to grow on unpolished rice. We even use the diet of oats and white bread to inhibit the growth of rats prior to feeding of the experimental diet. Further details on oats in relation to growth will be published later. Rats which are fed on oats autoclaved at 15 pounds' pressure die quickly, as is shown in Table VII.

TABLE VII.

Weight.		Duration.
Initial.	Final.	
gm.	*gm.*	*days*
105	66	20
76	59	6
103	56	9
140	80	21

SUMMARY.

The symptoms that develop in rabbits fed on oats are due possibly to acidosis and not to scurvy, judging from the beneficial effect of sodium bicarbonate and the ineffectiveness of the antiscorbutics. Guinea pigs on the same diet are not influenced by the alkali and respond so slightly to the action of antiscorbutics that the identity of this condition with human scurvy seems doubtful. Rats can live on oats for a considerable time, but not on autoclaved oats; young rats, however, fail to grow on this diet.

[11] Funk, *Proc. Roy. Soc. Med.*, 1913, vii, 9; *Z. physiol. Chem.*, 1913, lxxxviii, 352.

THE COMPOSITION AND PHYSIOLOGICAL ACTIVITY OF THE PITUITARY BODY. II.

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(Received for publication, May 5, 1916.)

Comparison of Activity of Adult and Infant Glands.

In a previous communication[1] it was shown that no distinct seasonal variation exists in the physiological activity and chemical composition of the pituitary body of cattle. Only glands from adult animals were investigated.

The material for the present work was selected from calves 2 to 4 months old, as well as from full grown cattle. None of the calves had been castrated or spayed, while a large majority of the mature animals were steers. Glands available for manufacturing purposes are obtained from the large abattoirs, and the majority of cattle slaughtered for human consumption are steers. With the exception of the sex glands and the thymus, the various organotherapeutic preparations are manufactured from glands obtained from castrated and non-castrated animals indiscriminately.

It has been pointed out that a high percentage of pituitary glands from cattle contain solid colloidal deposits between the anterior and posterior lobes at all four seasons. These deposits, which take the shape of the intralobular space and often expand the gland to the point of deformity, were found both in large and small glands, and no relation could be established between the size of the colloidal substance and of the gland. A number of pituitary bodies from young calves contained a light colored, opalescent, viscous liquid, but no solid colloidal material. The formation of solid colloids, therefore, is not a normal occurrence

[1] Fenger, F., *J. Biol. Chem.*, 1915, xxiii, 283.

during the growth period, but happens in the adult stage. The solid colloidal material is insoluble in all the ordinary solvents and is physiologically inert. It is evident, therefore, that the inspissated colloidal masses consist of the insoluble non-dialyzable portion of the pituitary secretion, which in the course of time has collected in such quantities as to be obstructive and undoubtedly of considerable hindrance to the normal functions of the gland.

The calf glands were collected from approximately 900 healthy, non-castrated calves, 2 to 4 months old, during December, 1915. The pituitaries from full grown cattle, representing many thousand animals, were obtained during the summer and fall of 1915. The method of collecting and preparing the glands for analytical purposes was identical with that described in detail in a previous communication.[1] In Table I are given the weights of the fresh glands and the yield of posterior lobe, also the moisture, the petroleum ether-soluble portion, and the yield of desiccated fat-free material. In the desiccated, fat-free, powdered material the moisture, ash, and total phosphoric acid were determined. Only the calf glands were submitted to chemical analysis, as various lots of the glands from mature animals have been analyzed and reported in a previous paper.[1]

TABLE I.

Lobes from Calves Collected December, 1915.

No. of glands	875	
Weight, gm.		
Average	0.7	
Maximum	1.1	
Minimum	0.4	
Yield of posterior lobe from whole glands, per cent.	12.6	
	Anterior.	Posterior.
	per cent	*per cent*
Moisture in fresh tissue	77.6	77.8
Petroleum ether-soluble	1.3	2.6
Desiccated fat-free material	21.1	19.6
Moisture	4.50	6.05
Ash	4.80	5.80
P_2O_5	2.68	2.65

These figures do not differ materially from those obtained from full grown cattle, except that the phosphoric acid is slightly higher, both in the anterior and posterior lobes of the calf glands.

The petroleum ether-soluble portion of the posterior lobe from pituitary bodies is a yellowish brown, waxy substance resembling crude phosphatides as obtained from brain tissue by the same solvent. It forms emulsions with water readily, but possesses no uterine-contracting power. A sample contained 4.74 per cent of P_2O_5, equivalent to 53.3 per cent of lecithin.

The physiological activity of the posterior lobe, both from calf and cattle glands was determined by the isolated uterus method as outlined by Roth.[2] The various pituitary solutions were made to represent a 5 per cent solution of the fresh posterior lobes. The glands were finely minced, macerated with slightly acidulated isotonic salt solution, heated to boiling, and filtered. The clear liquid was filled into ampules and sterilized. By mincing the glands exceedingly fine and by improved methods of extraction and filtration, solutions were produced which far surpassed in activity those prepared in this laboratory and reported a year ago.[1]

The standard was prepared by dissolving 0.1 gm. of β-imidazolylethylamine hydrochloride (hereafter referred to as β I) in 100 cc. of distilled water. This liquid was poured into ampules and sterilized at the same time and temperature as the lots of pituitary liquid. The standard as well as the various pituitary liquids were made in duplicate. A 1: 20,000,000 dilution of the standard in Locke's solution was maintained throughout the experiments. Various dilutions of the pituitary liquid were tried until the exact strength was found which matched the contraction produced by the standard. It may be mentioned here that the dilution of the standard in Locke's solution keeps well throughout the day, but the dilutions of pituitary liquid must be tested soon after they are made up as Locke's solution has a deteriorating effect on the uterine-contracting active principle, and solutions lose considerable strength on standing for 1 or 2 hours.

The selection of a suitable uterus is not always an easy matter. Often three or four guinea pigs have to be killed before a satis-

[2] Roth, G. B., *Bull. Hyg. Lab., U. S. P. H.*, No. 100, 1914.

factory strip is obtained. The individual sensitiveness towards pituitary and β I varies considerably. It is, consequently, only by comparing several tracings made on different days that a fairly correct conclusion in regard to the actual strength of a certain solution may be drawn. It would be desirable to obtain a standard which resembles more closely the active principle of the posterior lobe than does β I. In this laboratory a 2.5 cm. strip of the uterine end of one of the horns is employed. The ovarian end is discarded. The attachments are made by running a silk thread through the strip with the aid of a fine needle and then making a loop once around the entire strip and fastening with a knot. This prevents sagging of the uterus during the test and does not interfere with its sensitiveness. The silk attachments are made while the uterus is still in place in the animal. This saves time as well as unnecessary and undesirable handling of the strip. It generally takes from 1 to 1½ hours to adjust the uterus and balance it so that the rhythmical contractions are regulated to the proper size. Concordant and reliable results may then be obtained for 3 or 4 hours. After that time the uterus becomes hypersensitive and the tracings are consequently unreliable. Some uteri are rather slow in getting back to rhythmical contractions after the active solutions have been replaced by the blank, but in all instances this must be done before a fresh test solution is added. In case the guinea pig is light and the uterus thin, both horns may be tied together and the lower attachment fastened well into the body of the uterus.

In the present work determinations were made several times, on different days during the last few months, and it was found that the following dilutions of the original 5 per cent solutions of the posterior lobes were necessary to produce the same contractions as a 1: 20,000,000 dilution of the standard.

Posterior lobes from full grown cattle	1 : 38,000
" " " young calves	1 : 40,000

These figures indicate that the pituitary bodies from young animals are slightly more active than the glands from full grown cattle. This is in accordance with conditions found in other ductless glands, such as the thyroid, thymus, and suprarenals.

Observations on the Extraction of the Glands.

In order to determine the quantity of the water-soluble portion of the posterior lobe, 10 gm. portions of the fresh, finely minced lobes were extracted with water containing 0.5 per cent of glacial acetic acid, boiled for 10 minutes, cooled, made up to 100 cc., and filtered. If no acid is used a turbid solution results from colloidal suspension of lecithin and similar products, and it is impossible to filter the liquid clear. The filtrate after evaporation *in vacuo* to constant weight gave 0.691 gm. of total solids. In other words, 6.91 per cent of the fresh posterior lobe is soluble in acidulated water. The dry, acid water-soluble extractives possessed a uterine-contracting power of approximately one-half the strength of the crystalline β I. This residue forms yellow, hygroscopic, amorphous scales, and contains besides the uterine-contracting active principles, considerable amounts of phosphates and a high percentage of water-soluble organic extractives.

The uterine-contracting active principle may also be extracted by means of alcohol, as shown by the following experiments. Some finely minced fresh posterior lobes were extracted with neutral, and others with acidulated methyl or ethyl alcohol, using 100 gm. of glands and 200 cc. of alcohol. The glands were macerated with the solvents, heated to boiling, and filtered. The residues were washed with more solvent and the filtered liquids evaporated to dryness *in vacuo*. The dry residues thus obtained were exceedingly active. The acidulated methyl alcohol extract, for instance, showed a uterine-contracting power somewhat stronger than pure β I. This particular residue was of a yellowish brown color and contained lipoids, organic extractive matters, phosphates, etc. It was only partly soluble in water and it is certain, therefore, that the pure active principle of the posterior lobe must be very much stronger in uterine-contracting power than crystalline β I. The lipoids, although inert themselves, seem to be closely associated with and to exert a protective action upon the active principle. If, for example, the desiccated alcohol extract is redissolved in acidulated water in which the lipoids are insoluble, and the clear filtrate evaporated to dryness, the resulting extract is lower in uterine-contracting power than the original alcoholic extract. The active principle is apparently

susceptible to oxidation from the air. This is shown by the fact that solutions made from the desiccated fat-free gland are less active than those made from the fresh gland when proportionally equal amounts are employed.

SUMMARY.

The physiological activity of the posterior lobe of the pituitary body is somewhat higher during the growth period than after maturity. This is analogous to the conditions existing in the thyroid, the thymus, and the suprarenals. The infant gland contains more phosphates both in the anterior and posterior lobes than glands from fully mature animals.

The uterine-contracting active principle of the posterior lobe of the pituitary body is readily extracted from the fresh glands by water and also by neutral and acidulated methyl or ethyl alcohol. The acidulated methyl alcohol extract is more than twice as strong as the water extract and somewhat stronger than pure crystalline β-imidazolylethylamine hydrochloride.

THE RÔLE OF PSYCHIC AND SENSORY STIMULI IN THE HYPERGLYCEMIA PRODUCED BY LOWERING THE ENVIRONMENTAL TEMPERATURE OF DOGS.

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In 1845 Claude Bernard[1] discovered that glucose is present in normal blood. Since this discovery a large number of articles have appeared dealing (1) with methods for the quantitative estimation of the sugar content of the blood of various animals; (2) with the relative distribution of the sugar between the corpuscles and plasma; (3) with the nature of the reducing substances in blood; and (4) with the variations in the amount of sugar in the blood under physiological and pathological as well as under experimental conditions. This voluminous literature has been ably reviewed by Bang,[2] Macleod,[3] and Allen.[4]

Cannon and his collaborators[5] made the discovery that during strong emotion there occurs an increase in the percentage of sugar in the blood of cats synchronous with the appearance of a substance in the blood which inhibits the rhythmical contractions of the intestinal segment. Hirsch and Reinbach[6] subsequently found that a hyperglycemia of considerable degree may be produced if a dog is tied to a frame. These observations made it necessary to reinvestigate the work of earlier investigators with

[1] Bernard, C., Leçons sur le diabète, Paris, 1855, 175.

[2] Bang, I., Der Blutzucker, Wiesbaden, 1913.

[3] Macleod, J. J. R., Diabetes: Its Pathological Physiology, New York, 1913, 23.

[4] Allen, F. M., Studies concerning Glycosuria and Diabetes, Cambridge, 1913.

[5] Cannon, W. B., Shohl, A. T., and Wright, W. S., *Am. J. Physiol.*, 1911–12, xxix, 280.

[6] Hirsch, E., and Reinbach, H., *Z. physiol. Chem.*, 1914, xci, 292.

a keener appreciation of the importance of strong emotion and sensory stimulation as contributing factors that must be reckoned with in the interpretation of every form of hyperglycemia. More recently Bang[7] has reported that rabbits kept under laboratory conditions and on a laboratory diet for several weeks do not develop hyperglycemia when handled, or on being tied to a frame, as readily as do fresh animals. The literature on this subject has been reviewed by Cannon,[8] and also by Scott[9] who lays special emphasis on the importance of slight degrees of nervousness on the blood sugar of cats.

In 1907 Embden and his coworkers[10] reported that the glucose content of the blood of dogs varies inversely with the external temperature. In 1913 this work was repeated and confirmed by Silberstein[11] and the importance of the body temperature emphasized. Other investigators have obtained contradictory results.[12]

In our experiments we attempted to determine the rôle of sensory stimuli and strong emotion in the production of the hyperglycemia that followed the lowering of the environmental temperature of the dog.

Method of Procedure.

Animal.—The dog upon which these observations were made was a female bull terrier weighing 9 kg. at the beginning and 9.9 kg. at the end of the experiment. She was kept in the laboratory in a metabolism cage for 1 month and fed on a diet consisting of 250 gm. of meat, 200 gm. of bread, and 50 gm. of lard. The rectal temperature fluctuated from 38.4–39.2°C., and repeated examination of the urine even after the dog had been exposed to a temperature of 0–4°C. for 4 days, failed to show albumin or casts. The diet was given every afternoon at about 4 o'clock and the first sample of blood was collected in the morning during the post-absorptive period. The procedure of collecting the blood was repeated for over a

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[7] Bang, *Z. physiol. Chem.*, 1913, lxxxviii, 44.

[8] Cannon, W. B., Bodily Changes in Pain, Fear, and Rage, New York, 1915, 66.

[9] Scott, E. L., *Am. J. Physiol.*, 1914, xxxiv, 271.

[10] Embden, G., Lüthje, H., and Liefmann, E., *Beitr. chem. Phys. u. Path.*, 1907, x, 263.

[11] Silberstein, F., *Verhandl. deutsch. Kong. inn. Med.*, 1913, xxx, 93.

[12] Freund, H., and Marchand, F., *Arch. exp. Path. u. Pharm.*, 1913, lxxiii, 276.

month, so that the animal became thoroughly accustomed to it before the analyses were accepted.

A sample of blood was drawn at 10 a.m. and the room temperature was noted. The windows were then opened, the room was rapidly cooled, and blood was collected at intervals of ½, 4, and 6 hours. In some cases the cage was carried to a cold room. The room was then warmed and another sample of blood obtained. In subsequent experiments the animal was exposed to a warm environment for several days, then to a cold one for 4 days, and then again to a warm atmosphere for an equal period of time. Two and occasionally three samples of blood were drawn during the day at 4 and 6 hour intervals. All analyses are averages of duplicates which agree within 2 mg. of glucose in 100 cc. of blood.

Collecting the Blood.[13]—Hirsch and Reinbach drew blood in a similar manner. The dog's ear was shaved 1 or 2 days before and cleaned with alcohol and ether. When about to collect the blood the animal was gently lifted from the cage by the man who fed her, placed upon a table, and an incision was made into the edge of the ear with a sharp razor. The first few drops were allowed to flow away and subsequent drops were caught in a wide-mouthed bottle which contained a pinch of potassium oxalate powder. The application of a little pressure with a piece of sterile gauze or a piece of clean paper towel frequently sufficed to stop the flow, and when this was later removed blood again commenced to drip from the incision. In most cases the dog showed no discomfort during the entire procedure. Detailed notes were made as to the conduct of the dog while blood was being drawn and occasionally during the interim.

Method of Analysis.—The method used for estimating the sugar content of the blood was that of Lewis and Benedict,[14] the original technique being closely followed. A solution of Kahlbaum's pure *Traubenzucker* was used as a standard. This was restandardized from time to time. By drawing from 2 to 6 cc. of blood each time it was possible to analyze two or three specimens from the same sample and thereby obtain from four to six aliquots from each sample of blood collected. In order to test the accuracy of the method in our hands we analyzed specimens of blood of known sugar content to which definite quantities of the glucose standard had been added. Table I shows that glucose added to blood was recovered almost quantitatively. In view of the fact that but slight changes in blood sugar content were expected it was necessary to show that our margin of technical error was small. Duplicates and triplicates were therefore done on the same sample of blood and, as seen in Table II, our maximal variation is 2 mg. of glucose in 100 cc. of blood.

All colorimetric readings were made by one of us (K). Long[15] calls attention to the tendency of colorimeter prisms to slip down when the

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[13] The method of obtaining the blood was suggested to us by Dr. F. P. Underhill.

[14] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

[15] Long, J. H., *J. Am. Chem. Soc.*, 1916, xxxviii, 716.

external temperature is high, due to the liquefaction of the wax by means of which the prism is fixed in the collar, thereby introducing an error in reading the colorimeter. We were aware of this danger and therefore read the standard against itself both before and after each set of readings.

TABLE I.

Recovery of Glucose Added to Blood of Known Sugar Content.

Specimen.	Theoretical.	Found.	Difference.	
	mg.	*mg.*	*mg.*	*per cent*
51	3.365	3.412	0.047	1.37
53	3.287	3.300	0.013	0.39
58	3.159	3.216	0.057	1.7

TABLE II.

Margin of Error in Duplicates.

Specimen.	A.	B.	Specimen.	A.	B.
	mg.	*mg.*		*mg.*	*mg.*
64	0.087	0.087	79	0.081	0.083
65	0.094	0.095	84	0.091	0.089
67	0.105	0.104	82	0.096	0.098
76	0.098	0.096	85	0.083	0.083
77	0.097	0.097	86	0.089	0.087
78	0.092	0.091	87	0.084	0.087
				0.091	0.091

In Table III we have recorded the percentage of sugar in the blood, the room and rectal temperature, the day and hour that the blood was drawn; also whether or not it was necessary to make a new incision in order to get a sample of blood. In the last column the conduct of the dog during the collection of the blood is indicated. This table shows that in the absence of sensory and psychic stimulation there is no appreciable change in the percentage of sugar in the blood of a normal dog even when the animal is exposed to a room temperature of 2°C. for $3\frac{1}{2}$ hours and the environmental temperature fluctuates from 2–26°C. On the other hand, if by rubbing the incision the dog be made uncomfortable, considerable increase in the sugar content of the blood may be observed. When the blood sugar had risen to 0.104 per cent a new incision was made and the blood sugar content at

once began to drop in spite of the simultaneous fall in the external temperature, thus indicating clearly that the essential factor is not the external temperature but the emotional state of the ani-

TABLE III.

Date.	Specimen.	Glucose.	Temperature. Room.	Temperature. Rectal.	Hour.	Source, old or new incision.	Conduct.
1916		*per cent*	*°C.*	*°C.*			
Jan. 25	36	0.093	24	38.6	8.30 a.m.	Old.	Quiet.
" 26	37	0.090	14	38.8	2.10 p.m.	"	" unconcerned.
" 26	38	0.090	26	38.6	5.15 "	"	Resisted handling.
" 27	39	0.087	20.5	38.4	1.50 "	"	Quiet.
" 27	40	0.089	8.0	38.7	4.50 "	"	" unconcerned.
" 28	42	0.090	+ 2.0	38.8	12.00 m.	"	(Exposed to cold for 3½ hrs.) Quiet.
Average		0.089					
" 31	44	0.092	15.5	38.7	9.00 a.m.	New.	Struggled at first.
" 31	45	0.089	− 4	38.7	10.00 "	"	" "
" 31	46	0.100	0.0	38.7	3.00 p.m.	Old.	Excited by class in adjoining room.
Feb. 1	47	0.091	18	38.6	10.25 a.m.	"	Quiet. (Cage carried to cold room.)
" 1	48	0.093	+ 2	38.7	11.00 "	"	Whined a little.
" 1	49	0.096	− 3	39.2	3.00 p.m.	"	Shivered, otherwise quiet.
" 5	51	0.104	2.6	39.1	10.40 a.m.	"	Cut did not bleed freely; rubbed cut with gauze.
" 5	52	0.104	0.0	38.8	3.10 p.m.	"	
" 8	53	0.100	16	38.9	10.30 a.m.	"	Resisted slightly.
" 8	54	0.103	+ 3	39.1	11.25 "	"	
" 8	55	0.104	+ 3.7	38.8	2.45 p.m.	"	Restless.
" 9	56	0.100	+20.0	38.8	9.25 a.m.	New.	Ear appeared tender so a new cut was made. Quiet.
" 9	57	0.096	4.1	38.9	10.20 "	"	
" 9	58	0.094	4.7	38.8	2.40 p.m.	"	Quiet.
" 9	60	0.093	21.2	38.8	10.30 a.m.	Old.	"

mal. However, if a dog is kept in a cold environment for a period of 24 hours or more a hyperglycemia appears which is not to be attributed to sensory stimulation or the emotional state of the animal.

TABLE IV.

Effect on the Blood Sugar of Long Continued Exposure to Warmth.

Date.	Specimen.	Sugar.	Temperature.		Duration of exposure.	Source, old or new incision.	Conduct.
			Room.	Rectal.			
1916		*per cent*	*°C.*	*°C.*	*hrs.*		
Feb. 25	62	0.088	32	38.77	24	New.	Quiet.
" 25	63	0.088	33	38.83	26	Old.	"
" 25	64	0.087	31	38.72	29	New.	"
Mar. 6	78	0.091	25	38.66	43	"	Barked.
" 6	79	0.082	29.5	38.72	49	Old.	"
" 7	80	0.091	25.5	38.44	67	New.	Quiet.
" 7	81	0.091	29.0	38.5	71	Old.	"
" 8	83	0.089	29.2	38.44	93	New.	"
" 8	84	0.090	28.7	38.44	96	Old.	"
" 9	85	0.083	23	38.44	113	New.	
" 9	86	0.089	30	38.61	118	Old.	
" 9	87	0.083	31.5	38.66	120	"	Quiet.

The average percentage of blood sugar is 0.087.

TABLE V.

Effect on the Blood Sugar of Long Continued Exposure to Cold.

Date.	Specimen.	Sugar.	Temperature.		Duration of exposure.	Source, old or new incision.	Conduct.
			Room.	Rectal.			
1916		*per cent*	*°C.*	*°C.*	*hrs.*		
Feb. 26	65	0.095	5.4	38.50	17	Old.	Quiet, shivered.
" 26	67	0.104	4.1	38.55	21	"	" "
Mar. 1	68	0.107	7.0	39.00	48	New.	" "
" 1	69	0.106	7.0	38.88	50	Old.	" no shivering.
" 2	70	0.105	2.2	38.61	68	"	" shivered.
" 2	71	0.110	5.0	38.66	71	"	" "
" 3	72	0.097	7.0	38.66	100	New.	Shivered.
" 3	73	0.102	1.0	38.61	102	Old.	Quiet.
" 3	74	0.101	7.2	38.66	106	"	" no shivering.
" 3	75	0.109	4.0	38.88	108	"	No shivering.
" 4	76	0.097	0.2	38.55	125	"	Quiet, shivered.

The average percentage of blood sugar according to this table for continuous exposure to cold up to 125 hours is 0.102 per cent. The percentage increase in sugar as a result of long continued exposure to cold is 14.2 per cent, above the average obtained at room temperatures varying from 23–33°C., as shown in Table IV.

Weiland[16] found that muscular exercise, which does not produce a rise in body temperature and is not accompanied by respiratory distress, produces a fall in blood sugar, while Lusk[17] has succeeded in freeing the tissues of normal healthy men of their glycogen by immersion in cold baths that caused the individual to shiver. The question might be raised as to whether the increase in the percentage of sugar in the blood is due to cold alone or to shivering incidentally produced. The work of Weiland on the effect of muscular activity on blood sugar would lead one to expect the opposite effect; namely, a fall in blood sugar during shivering. Table V shows that the blood sugar was no higher when the dog was exposed to cold and shivered, than it was when she did not shiver, and in some cases it was even lower. Thus in Experiments 65 and 76, when the dog did shiver, the blood sugar was lower than the average for cold, while in Experiment 69, when the animal did not shiver, it was not lower than in the preceding or subsequent experiments when the dog did shiver. Specimen 72 is comparatively low, although the dog shivered, while Specimens 73 and 74 are much higher than the average at high temperatures, although the animal did not shiver in either case.

In the subsequent experiment the dog was exposed to a room temperature of 17°C. for 5 hours, at the end of which period, that is, at 3.45 p.m., a specimen of blood was drawn, and the blood sugar content found to be 0.085 per cent. The animal was quiet and not shivering. The room was then cooled to a temperature of about 12°C. The atmosphere was chilly and penetrating. The dog was exposed to this environment over night, and the next morning at 9.30 another sample of blood was collected. At this time the dog shivered. Duplicate analyses of this sample gave 0.084 and 0.086 per cent. The conclusion seems justified that shivering does not increase the percentage of glucose in normal dog's blood, but may decrease it.

[16] Weiland, W., *Deutsch. Arch. klin. Med.*, 1908, xcii, 223.

[17] Lusk, G., *Am. J. Physiol.*, 1910–11, xxvii, 427; 1908, xxii, 163.

CONCLUSIONS.

In the absence of adequate sensory and psychic stimulation the percentage of sugar in the blood of a normal dog did not vary when the animal was exposed to changes in external temperature for brief periods. A definite hyperglycemia followed the exposure of this animal to a low external temperature for a period of 24 hours or longer.

THE NATURE OF THE ACID-SOLUBLE PHOSPHORUS OF SERUM.

By ISIDOR GREENWALD.

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(Received for publication, May 22, 1916.)

In previous communications[1] the author has shown that phosphorus exists in the serum of dogs and of men in two classes of compounds: (1) phospholipins; (2) a form which is insoluble in the usual organic solvents but is soluble in a mixture of dilute acetic, or hydrochloric, and picric acids. The opinion was advanced that the latter was inorganic phosphate or a form closely related thereto. The question has been further investigated and the results obtained are presented in this paper.

In order to avoid the use of excessively large volumes, the serum was mixed with five volumes (instead of ten, as in previous work) of a 1 per cent solution of acetic acid saturated with picric acid. After standing 30 minutes, this was filtered and aliquots were taken for the following determinations.

A. Two portions of 500 cc. each were concentrated in a Kjeldahl flask, oxidized with sulfuric and nitric acids, and the phosphorus was precipitated as ammonium phosphomolybdate. This was filtered out, dissolved in ammonium hydroxide, the solution neutralized with citric acid, and the phosphate reprecipitated as ammonium magnesium phosphate.

B. Two portions of 500 cc. each were diluted with 300 cc. of water (to prevent subsequent precipitation of large amounts of ammonium picrate[2]), magnesia mixture and ammonium hydroxide were added, and the mixture was allowed to stand over night. The precipitate was then filtered out and washed with dilute

[1] Greenwald, I., *J. Biol. Chem.*, 1913, xiv, 369; 1915, xxi, 29; *Am. J. Med. Sc.*, 1914, cxlvii, 225.

[2] Ammonium picrate, though readily soluble in water, is quite insoluble in solutions of ammonium salts.

ammonium hydroxide, dissolved in cold citric acid, and reprecipitated by the addition of magnesia mixture and ammonium hydroxide.

C. Two portions of 500 cc. each were diluted with 300 cc. of water (to prevent subsequent precipitation of picric acid) and 125 cc. of molybdate solution[3] were added. A yellow precipitate appeared in a few seconds and settled slowly.[4] After standing at room temperature over night, it was filtered out, dissolved in dilute ammonium hydroxide, the solution neutralized with citric acid, and the phosphate reprecipitated with magnesia mixture.

Marriott[5] has reported a method for the determination of inorganic phosphate in small amounts of serum. He found from 1 to 3 mg., calculated as phosphorus, per 100 cc. of normal adult's serum. The inorganic phosphate was precipitated from the diluted serum by means of magnesia mixture. A similar technique was used in this work. Two portions of 100 cc. each of the serum were diluted with 200 cc. of water and treated with magnesia mixture and ammonium hydroxide. After standing over night the precipitate was filtered out, washed, dissolved in cold citric acid solution, and the phosphorus reprecipitated with magnesia mixture and ammonium hydroxide. The first precipitate could not be used directly as it contained considerable foreign material.

The precipitates obtained by any of these procedures were filtered on Gooch crucibles, washed with dilute ammonium hydroxide, dried in a water-jacketed oven, weighed, dried at 105° (Freas oven), weighed, and finally ignited and weighed. This was done to establish the constitution of the precipitate as ammonium magnesium phosphate and also to obtain data on the advisability of the routine weighing of the precipitate without

[3] This was prepared by dissolving 100 gm. of molybdic acid (85 per cent) in a mixture of 155 cc. of concentrated NH_4OH and 300 cc. of H_2O, and adding this to a mixture of 530 cc. of concentrated HNO_3 and 824 cc. of H_2O.

[4] The precipitate obtained by adding molybdate solution to a phosphate solution containing picric acid appears to be quite different from the usual ammonium phosphomolybdate. It was not analyzed, but one striking difference is the ease with which it dissolves in alcohol. It appears to contain picric acid.

[5] Howland, J., Haessler, F. H., and Marriott, W. McK., *J. Biol. Chem.*, 1916, xxiv, p. xviii; also personal communication to the author.

ignition.[6] The results indicate that, after drying at 95–100°, the precipitate has the composition $MgNH_4PO_4.\frac{1}{2}H_2O$ and that the remainder of the water is driven off when the precipitate is dried at 105°. These assumptions were made in calculating the figures given in the tables.

Because of the small amount of material available, the total "acid-soluble" phosphorus in some of the specimens was determined by the colorimetric method described by the author.[7] In one case the inorganic phosphate was determined by titration of the reprecipitated ammonium phosphomolybdate.

The results obtained are summarized in Table I and clearly indicate that practically all the phosphorus extracted from serum

TABLE I.

Serum Phosphorus, as Determined by Different Methods, in Mg. per 100 Cc. of Serum.

Serum from	Total acid-soluble phosphorus.			Phosphorus precipitated by Mg mixture in acid extract.			Phosphorus precipitated by molybdate solution.			Phosphorus precipitated by Mg mixture in serum.		
	100°.	105°.	Ignited.	100°.	105°.	Ignited.	100°.	105°.	Ignited.	100°.	105°.	Ignited.
Sheep.....	11.00	11.13	10.91	10.89	10.53	10.48	10.31	10.30	10.32	10.37	9.89	9.84
"	12.01	11.57	12.04	11.40	10.88	10.98						11.88
"	8.58		8.60		8.13	8.04	7.66		7.43	7.56		7.58
Beef.....		6.79	7.04				7.01	7.25	6.97	6.31	6.34	6.35
Pig.....	9.70	9.53	9.55			9.09	9.58	9.35	9.38	9.09	9.30	9.02
Dog.....		5.51*			5.62	5.58						
Human.												
M. B....		4.74*								4.31	4.17	4.18
I. G.....		2.61*						2.94†				
Chest												
fluids....		5.05	4.74								3.56	3.48
		3.83	3.64					2.92	3.01		3.28	3.18
Abdominal												
fluid.....		2.59	2.25					2.08	2.28		2.45	2.29

* Determined colorimetrically.

† Determined by titration of the reprecipitated phosphomolybdate.

[6] The paper by Jones (*J. Biol. Chem.*, 1916, xxv, 87), in which the results obtained by weighing the air-dried precipitate, $MgNH_4PO_4.6H_2O$, are described, appeared after this work had been completed and the results prepared for publication.

[7] Greenwald, *J. Biol. Chem.*, 1915, xxi, 29.

by dilute acid was in the form of inorganic phosphate or else an extremely labile organic compound. If the latter, it was so unstable that it is extremely difficult to obtain evidence of its existence.

There was, however, a slight difference between the amount of total acid-soluble phosphorus and that precipitated by magnesia mixture or by molybdate solution. The following experiments were performed to ascertain whether or not this difference might be due to the presence of substances that interfered with the precipitation of inorganic phosphate.

A measured volume of serum was dialyzed for 4 or 5 days and the volume again measured. It still contained about 0.3 mg. of acid-soluble phosphorus per 100 cc. of serum. The equivalent of each 100 cc. of the original serum was mixed with a solution containing 0.8 gm. NaCl, 50 mg. urea, 0.1 gm. glucose, 10 mg. creatine, and 7.85 mg. phosphorus (as K_2HPO_4). This liquid was then treated in the manner already described. From the results summarized in Table II it is evident that the discrepancies obtained in the first series of experiments cannot be explained on the basis of interference with precipitation by substances in this semi-artificial serum. It is, of course, possible that serum contains some other substance which interferes with the quantitative precipitation of inorganic phosphate. This is not likely and it would seem that phosphorus may occur in a form other than inorganic phosphate and phospholipins. However, the amount of such phosphorus is very small and does not exceed 1 mg. per 100 cc. of serum.

TABLE II.

Recovery of Phosphate Added to Dialyzed Serum, in Mg. per 100 Cc. of Serum.

Serum from	Acid-soluble phosphorus in dialyzed serum.	Phosphate added as phosphorus.			Phosphorus precipitated by molybdate solution.			Phosphorus precipitated by Mg mixture in serum.	
		100°.	105°.	Ignited.	100°.	105°.	Ignited.	105°.	Ignited.
Pig.....	0.30*	7.84	7.75	7.85					7.87
Sheep.....	0.28*	7.84	7.75	7.85	7.50	7.50	7.53	7.52	7.62

* Determined colorimetrically.

SUMMARY.

The phosphorus compounds of serum consist almost exclusively of phospholipins and inorganic phosphate. There is some indication of the presence of a form which is soluble in dilute acids but is not precipitated by magnesia mixture or by molybdate solution, and which does not dialyze readily from serum.

THE CHEMICAL NATURE OF THE "VITAMINES."

I. ANTINEURITIC PROPERTIES OF THE HYDROXYPYRIDINES.*

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The difficulties involved in the isolation of "vitamines" from yeast or rice polishings have proven so serious that success seemed doubtful until a clearer idea should be gained of the chemical nature of these important substances. Accordingly attention has been directed to the preparation and testing of synthetic substances, which appeared likely to display some curative properties similar to the "vitamine fraction" of natural foodstuffs.

Two facts were considered of primary importance in determining the direction of the work. The first of these was that the curative substance seemed to be a pyridine derivative since the curative fraction of rice polishings contains nicotinic acid,[1] and has been found to develop a pyridine-like odor under certain conditions. A second hint was found in the fact that the blue color reaction given by antineuritic foodstuffs when treated with phosphotungstic acid and alkali seems to be rather closely associated with the potent constituents.[2] Since this reaction and the similar reaction with phosphomolybdic acid[3] are known to be

* A considerable portion of the work here recorded was reported in a paper presented at the Second Pan-American Scientific Congress at Washington, D. C., Jan. 3, 1916, the Proceedings of which have not yet been published.

[1] Suzuki, U., Shimamura, T., and Odake, S., *Biochem. Z.*, 1912, xliii, 89. Drummond, J. C., and Funk, C., *Biochem. J.*, 1914, viii, 598, and elsewhere.

[2] Drummond and Funk, *Biochem. J.*, 1914, viii, 598. Folin, O., and Macallum, A. B., Jr., *J. Biol. Chem.*, 1912, xi, 265; 1912–13, xiii, 363.

[3] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xii, 239. Funk, C., and Macallum, A. B., Jr., *Biochem. J.*, 1913, vii, 356.

produced by substances containing hydroxyl groups in the benzene ring, it was not surprising to find that hydroxyl derivatives of pyridine also give one or both reactions according to the number of the substituted hydroxyl groups.

Accordingly a series of pyridine derivatives was prepared and the therapeutic action of each individual roughly tested on polyneuritic pigeons by the administration of doses of 1 to 10 mg. by intramuscular injection. In some cases doses of 10 to 100 mg. were administered by mouth as a supplementary test. The series included nicotinic, cinchomeric, quinolinic, 6-hydroxynicotinic and citrazinic acids, α-hydroxypyridine, glutazine, 2,4,6-trihydroxypyridine and its anhydride, and finally 2,3,4-trihydroxypyridine and the so called tetrahydroxypyridine.

Nicotinic, cinchomeric, and quinolinic acids, were prepared by the oxidation of nicotine, quinine, and quinoline respectively;[4] and citrazinic acid from citric acid.[5] The methods of Pechmann and Stokes[6] were followed for the preparation of ethylamino-β-hydroxyglutamate, glutazine, 2,4,6-trihydroxypyridine and its anhydride. Tetrahydroxypyridine and 2,3,4-trihydroxypyridine were produced by the method of Ost[7] from the nitroso derivative. In the distillation of meconic acid for this purpose the modification of Peratoner and Leone[8] was used. Better methods than those of Ost are offered by Peratoner and Castellana[9] for the production of hydroxycomenic acid from which by suitable modification of Ost's second method, fair yields of 2,3,4-trihydroxypyridine may be obtained. These methods are being studied as are those of Collie,[10] Tickle and Collie,[11] Lapworth and Collie,[12] and Baron,

[4] Weidel, J., *Ann. Chem.*, 1873, clxv, 330. Weidel, H., and v. Schmidt, M., *Ber. chem. Ges.*, 1879, xii, 1146. Hoogewerff, S., and Van Dorp, W. A., *Rec. trav. chim. Pays-Bas*, 1882, i, 107.

[5] Behrmann, A., and Hofmann, A. W., *Ber. chem. Ges.*, 1884, xvii, 2687.

[6] v. Pechmann, H., and Stokes, H. N., *Ber. chem. Ges.*, 1885, xviii, 2291. Stokes, H. N., and v. Pechmann, H., *ibid.*, 1886, xix, 2694.

[7] Ost, H., *J. prakt. Chem.*, 1879, xix, 203; 1883, xxvii, 257.

[8] Peratoner, A., and Leone, R., *Gazz. chim. ital.*, 1894, xxiv, pt. ii, 75.

[9] Peratoner, A., and Castellana, V., *Gazz. chim. ital.*, 1906, xxxvi, pt. i, 21.

[10] Collie, J. N., *J. Chem. Soc.*, 1891, lix, 617.

[11] Tickle, T., and Collie, J. N., *J. Chem. Soc.*, 1902, lxxxi, 1004.

[12] Lapworth, A., and Collie, J. N., *J. Chem. Soc.*, 1897, lxxi, 838.

Remfry, and Thorpe.[13] Coumalic acid was used for making hydroxynicotinic acid,[14] by the distillation of which α-hydroxypyridine is readily obtained.[15]

The test animals used were pigeons, in which polyneuritis was developed by feeding *ad libitum* on white rice. In all cases the disease ran its course till the animals were unable to stand or make controlled movements of the legs or wings. In such cases death usually follows within 24 hours and may occur at any moment. A number of times birds died in the experimenter's hands before treatment could be administered. During and after treatment the birds were continued on a diet of white rice, and under such conditions a redevelopment of the symptoms can be only a matter of a few days. Since forced feeding was not resorted to on account of the time necessary to care for a large number of birds in this way, it was occasionally the case that the pigeons became weak and emaciated before the characteristic symptoms developed to the desired point. Such cases are not of course strictly comparable with those of sudden development in which the animals retain a great deal of their original strength and vigor, though losing muscular control, and a measure of uncertainty is thereby introduced in occasional cases.

On treating polyneuritic pigeons with the substances above mentioned definite evidence of curative power was noted in the case of α-hydroxy-, 2,4,6-trihydroxy-, and 2,3,4-trihydroxypyridine. The remainder of the series showed no effect. But as will be seen later, such negative evidence in the case of any hydroxypyridine derivative cannot be regarded as conclusive. The first of the curative substances tested was α-hydroxypyridine. Three birds were treated with excellent results. However, three others treated later showed little or no improvement. On proceeding with the series to the polyhydroxy compounds, a rapid striking cure was obtained with a preparation of 2,4,6-trihydroxypyridine, followed by several partial or complete failures. A second and third fresh preparation, however, produced two and three fairly rapid cures respectively. A single fresh preparation

[13] Baron, H., Remfry, F. G. P., and Thorpe, J. F., *J. Chem. Soc.*, 1904, lxxxv, 1726.

[14] v. Pechmann, H., and Welsh, W., *Ber. chem. Ges.*, 1884, xvii, 2384.

[15] v. Pechmann, H., and Baltzer, O., *Ber. chem. Ges.*, 1891, xxiv, 3144.

of 2,3,4-trihydroxypyridine was administered to three pigeons simultaneously at the close of the working day, one pigeon receiving 2, the second 1, and the third 0.5 mg. The following morning the first bird was dead, while the second and third had completely recovered from all paralytic symptoms. Subsequently the same material failed to benefit several birds to which it was administered.

In each case all the cures obtained were of those pigeons which were first treated with a given preparation, while those treated with the same preparation a few days or weeks later invariably received no benefit. It was obvious that the substances had changed in some manner so as to lose the curative power. As there was no evidence of decomposition, it seemed probable that it was due to isomerisation. Such rearrangements have greatly interested organic chemists for many years, but no definite evidence has been adduced that such transformations take place in the tissues as a fundamental part of a biological process, though they appear to be just such reactions as might easily occur within the living organism. Many of them take place with the greatest ease and without resort to the vigorous methods ordinarily employed in our laboratories.

The existence of a tautomerism in the hydroxypyridines has long been recognized in a general way. It has been observed that these substances under certain circumstances react as hydroxy or enol compounds, under others as ketones. However, no one has regarded it possible that two isomers could exist in a free isolated state as two distinct individuals. Each of the hydroxypyridines is described in the literature as one substance of definite though dual properties. A critical examination of the literature, however, suggested that this was not the case. It was therefore decided to study the isomerism of some one of the curative substances more thoroughly than had been done previously. For this purpose α-hydroxypyridine was chosen. This substance has been administered to about eighty pigeons under varying conditions and certain important facts have come to light.

It is prepared by fusing 6-hydroxynicotinic acid and distilling the residue after the evolution of carbon dioxide has ceased. The oily distillate presently solidifies to a mass made up of either granular or needle crystals, or both, depending apparently on the

rate of cooling and the presence or absence of the merest traces of moisture. The residue condensing in the neck of the distilling flask after completion of the process always crystallizes in the form of needles. A crystalline granular mass so obtained on being melted and kept at a temperature well above the melting point for some time and allowed to cool slowly, again solidifies to a mass, now consisting largely of needles. If this remelted mass is dissolved in benzene and recrystallized therefrom by the addition of ligroin, needles are found to predominate in the beautiful crystalline precipitate. Sometimes the needles may be obtained absolutely free from granules. From the mother liquor on concentration, granular crystals separate and may be obtained in a pure form by filtering from the hot solvent, as the small residuum of needles dissolves freely in the hot mixture of ligroin and benzene. Both forms after washing freely with petroleum ether and drying rapidly in a vacuum desiccator, melt sharply at 106–107°. A fused mixture does not melt sharply and the melting point varies with the speed with which the temperature is raised. Evidently granular crystals are converted partially into needles by dry heat. On allowing the solid needle form to stand for a few days in an open vessel or cork-stoppered bottle, it will be found that the crystal form has undergone a change. The needles which were originally clear and sharply defined are marked by transverse lines of cleavage. In the course of 10 to 20 days they become roughly needle-shaped granular aggregates. The rate of this change is variable but seems to depend greatly on the amount of moisture in the atmosphere surrounding the crystals. By exclusion of moisture the transition takes place much more slowly. A water solution of needles on evaporation deposits only granules which, however, may be partially reconverted into needles by dry heat.

If the two forms of crystals are titrated in the cold with alcoholic bromine according to the method of Kurt Meyer,[16] neither absorbs appreciable amounts of bromine instantaneously. The granular form absorbs bromine gradually and the needle form still more slowly. Neither form can therefore be the hydroxy or enol form. However, on dissolving either form in an excess of

[16] Meyer, K. H., *Ann. Chem.*, 1911, cccIxxx, 212.

alcoholic sodium hydroxide of known strength, and titrating in the cold with bromine, instantaneous absorption occurs in excess of that required by the caustic soda. In the presence of from five to ten molecular equivalents of sodium hydroxide, from 50 to 60 per cent of the theoretical quantity of bromine is absorbed with great rapidity. α-Hydroxypyridine in alcoholic sodium hydroxide solution is unquestionably largely, though not wholly, in the enol form. Presumably all the metallic salts are likewise enols, a presumption which is in accord with the known facts in regard to other enol-keto tautomers. If the cold alcoholic solution of the sodium salt is just neutralized with cold alcoholic hydrochloric acid detectable amounts of the free enol form are momentarily present as determined by bromine titration. The existence of any considerable proportions of the enol form in the free state appears to be brief in all neutral solvents and no method has been found for its isolation. Since α-hydroxypyridine is a base as well as an acid, it forms well defined salts with strong acids. Whether these salts are partly salts of the enol form cannot be determined by titrimetric methods, since the presence of the acid makes the determination of an end-point impossible. If these salts are not partly enols they are at least transformed into enols with the greatest rapidity during titration.

It appears that there exist not two but three isomeric forms of α-hydroxypyridine for which we may readily imagine structural formulas[17] expressing intramolecular rearrangement. This matter will be taken up in a later paper. By simple means we may cause any desired form to predominate and may pass through the cycle repeatedly. Each exists in equilibrium with at least one other in relative quantities depending on conditions. We shall understand their relationships fully only after finding a method for determining each of the three forms quantitatively under varying conditions. At present we can measure only the amount of enol.

To a degree it has been possible to determine the curative power of each of the three forms. Several preparations of freshly crystallized needles dissolved in water immediately before injection have been administered to fourteen polyneuritic pigeons in

[17] Kauffmann, H., *Ber. chem. Ges.*, 1903, xxxvi, 1062. Decker, H., *J. prakt. Chem.*, 1900, lxii, 266.

doses of 1 to 2 mg. Improvement or cure resulted in every case as evidenced by change in weight, paralytic symptoms, and appetite. Eight of the birds reached an optimum from 1 to 3 days after treatment and after a single dose of 1 to 2 mg. lived from 5 to 10 days. Three birds were partially cured a second and third time after redevelopment of severe symptoms and lived 11, 13, and 15 days respectively after the first onset of the disease. However, six cures differed markedly from the others obtained with α-hydroxypyridine. The first evidences of improvement were noticed in these cases in 40 minutes to 2 hours after injection, when the animals began to assume nearly normal attitudes. At intervals brief spasms of spastic movement occurred, which gradually became less frequent and less prolonged and in the course of 3 to 12 hours ceased altogether. After this time the birds did not differ markedly in condition from those in which less rapid cures were obtained. The gain in weight and other evidences of improvement were of the same character. The rapid cures obtained with 2,4,6-trihydroxypyridine above reported were attended by the same symptoms. In general it may be said that the results obtained with these substances approximate very closely those obtained with hydrolyzed extract of rice polishings, in both rapidity and completeness of action on the paralytic symptoms. The ultimate effects on the general health and weight of the birds were less satisfactory than those of the natural "vitamines." Protective experiments on healthy birds on a white rice diet have not yet been made. Water solutions of the needle form kept at ordinary temperatures retain some curative power for 5 or 6 days, after which it is no longer detectable. At boiling temperature the curative power disappears very rapidly.

The physiological effect of the enol form can only be guessed, as long as it remains impossible to obtain it free from the other forms and from extraneous substances. Injections of 1 mg. in caustic soda solution produced fairly rapid cures in two birds so treated. Administration by mouth of 10 mg. in caustic soda followed by dilute acetic acid also effected fairly rapid cures. The silver salt was also administered to three pigeons in doses of 20 mg. each followed by salt solution as an antidote for the silver. Two had recovered from the paralysis the following day and lived 3 and 7 days respectively. One died 5 hours after treatment.

No very satisfactory conclusion can be drawn from these experiments on account of the disturbing factor of the toxicity of caustic soda and silver. The former produced severe inflammation of the tissues with which it came in contact and the latter evidenced a general toxic action, as was to be expected. Nevertheless there was indubitable relief of the typical paralytic symptoms. This fact that solutions of α-hydroxypyridine in caustic soda are curative led to much confusion, as it suggested that the curative form was the enol. However, since hydroxypyridine under such conditions is only partly enol, as indicated by bromine titration, the curative property of caustic soda solution may be due to the non-enolic portion.

The granular form as obtained by prolonged standing of the needle form has been tested on some thirty birds under varying conditions. Doses of from 0.5 to 100 mg. have been tried by injection and by mouth. Injections in water have been made immediately after solution, after standing several days, after boiling, and after heating under steam pressure. Injections in mineral oil and in fatty acid have also been tried. In no case was there the slightest evidence of any benefit. When large doses were administered, the action seemed distinctly adverse in several cases, as the birds died sooner than was expected. Doses of 10 mg. injected into healthy birds, however, produced no gross toxic symptoms.

This absolute lack of curative properties is striking and suggestive. If the isomeric forms exist in equilibrium and there is fairly rapid transformation in any direction, one would suppose that the granular form would, when injected into the body, rapidly convert itself into the curative form as the latter was removed from solution by absorption in the tissues or fluids requiring it. That such is not the case suggests strongly that the pathological conditions of polyneuritis are not due to a deficiency of a substance *per se*, but to a lack of a certain type of potential energy which only certain substances can supply. In other words, one is led to surmise that it is the potentiality of isomeric change that produces the desired result. In this connection it is interesting to note that many purine and pyrimidine derivatives, some of which Funk has reported to be partially curative for poly-

neuritis,[18] are also theoretically capable of a similar isomerism. That these substances are to a greater or less degree enol-keto tautomers is strongly indicated, by well known reactions of uric acid for example. The existence of a third isomer is not excluded by theoretical considerations, but lacks the support of any considerable experimental evidence.

It appeared not unlikely that other hydroxypyridines might prove more easily controlled. Accordingly β- and γ-hydroxypyridine and γ-lutidone have been prepared and are being studied. They show curative properties only under certain conditions, and these curative properties automatically disappear with the lapse of time. Each crystallizes in two different forms and forms metallic salts which absorb bromine rapidly in cold alcohol. Present evidence indicates that they are strict analogues of α-hydroxypyridine.

The antineuritic properties of these substances suggest that an isomerism is at least partially responsible for the instability of the "vitamines" in foodstuffs, and that the antineuritic property may be inherent in the potentiality of this type of isomerism. We may not conclude that "vitamines" are necessarily hydroxypyridines since a similar isomerism may exist in substances containing other heterocyclic nitrogenous nuclei which are known to occur widely as constituents of animal tissue.

[18] Funk, C., *J. Physiol.*, 1912–13, xlv, 489.

STUDIES IN CARBOHYDRATE METABOLISM.

XI. THE RÔLE OF CALCIUM IN THE REGULATION OF BLOOD SUGAR CONTENT.

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A perusal of the literature gives one the impression that calcium salts may play a more or less important rôle in the regulation of carbohydrate metabolism even though their possible function is recognized only in a vague, indefinite manner. On the one hand, Bunge[1] has put forth a warning against a sugar-rich diet deficient in salts, especially iron and calcium, the inference being that such a deficiency may have an influence on the production of diabetes. This is especially pertinent in view of the fact that diabetes is said[2] to be associated with an increased output of calcium. Again, it is well known that the glycosuria evoked by long continued injection of sodium chloride into the blood may be checked promptly by the intravenous administration of dilute solutions of calcium chloride.[3] On the other hand, the introduction into the blood of strong[4] solutions of calcium chloride is sufficient to induce the appearance of sugar in the urine.

Schrank[5] has claimed that calcium salts will either prevent or inhibit the glycosuria called forth by epinephrin.

Calcium salts have also been associated closely with the function of the thyroid-parathyroid mechanism, MacCallum and

[1] v. Bunge, G., *Z. Biol.*, 1901, xli, 155.

[2] Compare Allen, F. M., Studies concerning Glycosuria and Diabetes, Boston, 1913.

[3] For literature see Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1905-06, xv, 321.

[4] Underhill, F. P., and Kleiner, I. S., *J. Biol. Chem.*, 1908, iv, 395.

[5] Schrank, F., *Z. klin. Med.*, 1909, lxvii, 230.

Voegtlin[6] having demonstrated a loss of calcium to the body after extirpation of this apparatus together with cessation of tetany symptoms subsequent to calcium injections. Underhill and Blatherwick[7] showed that during the tetany attendant upon thyreoparathyroidectomy the blood sugar content is markedly diminished, and in a later paper[8] evidence was presented, confirming the results of MacCallum and Voegtlin, that calcium checks tetany, and furthermore, demonstrating that the blood sugar content likewise may be restored to normal temporarily by calcium administration. It was stated:

"It is apparent from these results that in tetany calcium plays an important rôle in maintaining the equilibrium of the sugar-regulating mechanism, for when there is a sufficiency of calcium blood sugar content is normal. Conversely, blood sugar content tends to become low when there is an apparent deficiency of calcium."[9]

The data of the present communication were obtained in an endeavor to determine the influence which calcium salts may exert upon the blood sugar content, first, under normal conditions, and, second, under circumstances in which the equilibrium of the blood sugar-regulating mechanism had been disturbed experimentally. For the latter purpose epinephrin was employed.

Methods.

Normal full grown rabbits maintained under practically uniform conditions served as experimental animals. Only a single experiment was performed upon each animal. This condition was enforced because of the well known fact that epinephrin may fail to elicit the maximum effect subsequent to the initial injection. The epinephrin employed was the adrenalin of Parke, Davis and Company, 1 : 1,000. Maximum doses, 1 cc. (1 mg.) per kilo of body weight, were administered. Blood sugar was estimated by the Forschbach and Severin[10] method, which has been found to yield results in good agreement with the procedure of Waymouth Reid.[11] Sugar in the urine was determined gravimetrically by the Allihn method.

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[6] MacCallum, W. G., and Voegtlin, C., *J. Exp. Med.*, 1909, xi, 18.

[7] Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xviii, 87.

[8] Underhill and Blatherwick, *J. Biol. Chem.*, 1914, xix, 119.

[9] Underhill and Blatherwick, *J. Biol. Chem.*, 1914, xix, 125.

[10] Forschbach and Severin, *Arch. exp. Path. u. Pharm.*, 1912, lxviii, 341.

[11] Reid, E. W., *J. Physiol.*, 1896, xx, 316.

The Influence of Subcutaneous Injections of Calcium Lactate and Calcium Chloride upon Blood Sugar Content.

From the data in Table I it may be seen that the subcutaneous administration of small or large doses of calcium salts (lactate and chloride) is without significant influence upon the blood sugar content under the experimental conditions recorded.

TABLE I.

The Sugar Content of the Blood after Subcutaneous Injections of Calcium Lactate and Calcium Chloride.

Rabbit.	Body weight.	Blood sugar content (in percentages).							Remarks.
		Normal.	Hours after calcium injection.						
			1	2	3	4	5	6	
	gm.								
22	2,200	0.16	0.17	0.17	0.13	0.11	0.15	0.16	Injection of 10 cc. 3 per cent calcium lactate.
23	2,200	0.12	0.15	0.17	0.16	0.10	0.15	0.15	
26	2,000	0.12	0.10	0.12	0.13	0.15	0.12		Injection of 20 cc. 3 per cent calcium lactate.
27	2,300	0.11	0.12	0.15	0.12	0.12	0.12		Injection of 30 cc. 3 per cent calcium lactate.
76	2,400	0.14	0.15	0.14	0.17	0.12	0.12	0.11	Injection of 25 cc. 11 per cent calcium chloride.
77	2,400	0.15	0.14	0.11	0.14	0.11	0.11	0.13	
Average...		0.13	0.14	0.14	0.14	0.12	0.13	0.14	No reducing substance in any urine.

CHART 1. Curve showing the sugar content of the blood after injections of calcium lactate and calcium chloride. Average of four experiments (Table I).

The Sugar Content of the Blood and Urine under the Influence of Epinephrin.

It is a well known fact that the same dose of epinephrin may not produce the same degree of glycosuria in two different animals or in the same animal at different periods. Therefore it is decidedly difficult to establish a standard which may be accepted as an average amount of sugar appearing in the urine under the stimulus of a given dose of epinephrin. With these limitations in mind four animals were taken at random from stock, all were given the same quantity of epinephrin per kilo, and the average amount of sugar excreted by the kidneys was accepted as the standard for epinephrin glycosuria. In like manner blood sugar estimations carried through at hourly periods were averaged for the same four animals. From the figures obtained a curve, Chart 2, has been plotted which represents the blood sugar changes subsequent to epinephrin treatment. As may be seen from Table II, the extent of glycosuria and the degree of hyperglycemia are distinctly variable, but the average figures are undoubtedly a fair representation of epinephrin glycosuria and hyperglycemia. A glance at the chart will show clearly that the hyperglycemia attains its maximum between the 3rd and 5th hours, gradually decreasing and approaching normal again at about the 8th hour. Approximately 1.5 gm. of sugar represent the average output under the influence of 1 mg. of epinephrin per kilo of body weight.

TABLE II.

The Sugar Content of the Blood and Urine under the Influence of Epinephrin.

Rabbit.	Body weight.	Blood sugar content (in percentages).							Sugar in urine.
		Normal.	Hours after epinephrin injection.						
			1	2	3	5	6	7½	
	gm.								*gm.*
3	2,100	0.13	0.27	0.40	0.50	0.27	0.30	0.21	0.46
4	2,000	0.10	0.50	0.49	0.42	0.40	0.36	0.28	1.58
5	2,000	0.12	0.30	0.39	0.53	0.40	0.26	0.12	1.58
6	2,000	0.18	0.36	0.43	0.41	0.43	0.36	0.16	2.34
Average.....		0.13	0.36	0.43	0.46	0.37	0.32	0.19	1.49

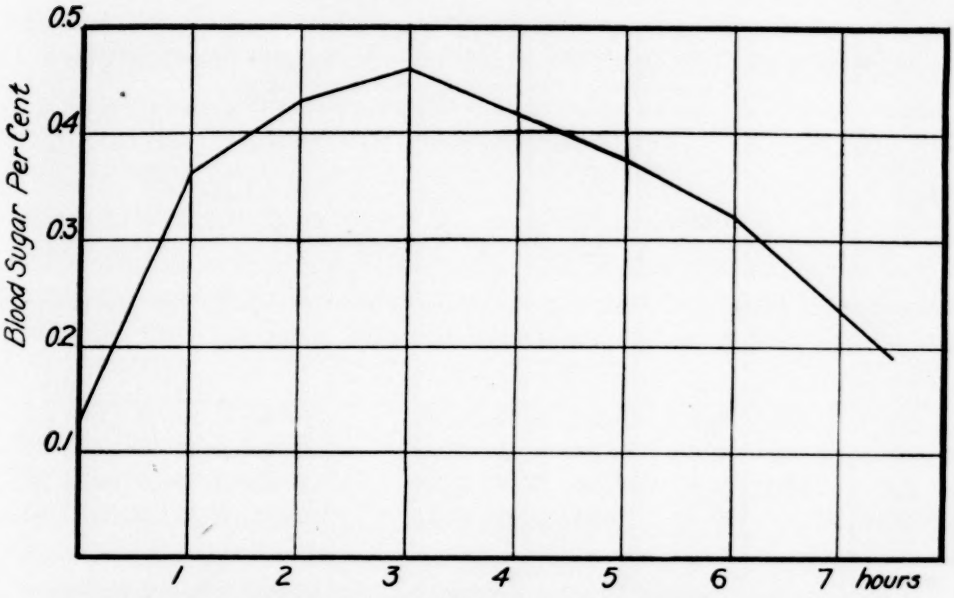

CHART 2. Curve showing the sugar content of the blood after injections of epinephrin. Average of four experiments (Table II).

The Influence of Various Doses of Calcium Salts upon the Hyperglycemia and Glycosuria Provoked by Epinephrin.

As far as I am aware the only investigation of the influence of calcium salts upon epinephrin glycosuria is that reported by Schrank.[5] He made determinations of the sugar in the urine only and claimed that calcium chloride markedly diminishes, or may entirely inhibit, epinephrin glycosuria. In his experiments the animals were interchanged in some instances; that is, those rabbits receiving epinephrin alone were employed later for experiments in which both epinephrin and calcium were given, and conversely. Relatively small quantities of epinephrin were administered, 0.8 to 1.0 cc. per animal, body weight varying from 850 to 2,720 gm.

Throughout this investigation the calcium salts were injected subcutaneously at varying periods previous to epinephrin administration in order to obtain the maximum influence of the calcium salt which is presumably rather difficult of absorption. The influence which calcium exerts upon epinephrin glycosuria and hyperglycemia is to be seen in Table III and Charts 3, 4, 5, 6, and 7, given below. In the first series of experiments, Table III,

TABLE III.

The Influence of Various Doses of Calcium Salts upon the Hyperglycemia and Glycosuria Provoked by Epinephrin.

Rabbit.	Body weight.	Blood sugar content (in percentages).								Sugar in urine.
		Normal.	Hours after epinephrin injection.							
			1	2	3	4	5	6	7½	
Subcutaneous injection of *10 cc. 1 per cent calcium chloride ½ hour* previous to epinephrin.										
	gm.									*gm.*
8	2,000	0.13	0.32	0.37	0.30		0.29	0.19	0.11	3.03
9	2,300	0.16	0.33	0.41	0.39		0.33	0.27	0.22	2.08
10	2,200	0.11	0.34	0.44	0.54		0.43	0.29	0.14	3.28
Average..		0.13	0.33	0.41	0.41		0.35	0.25	0.16	2.80
Subcutaneous injection of *10 cc. 1 per cent calcium lactate 2 hours* previous to epinephrin.										
13	1,800	0.15	0.54	0.52	0.37		0.30	0.19	0.15	2.83
14	2,200	0.15	0.54	0.53	0.39		0.25	0.18	0.14	5.20
Average..		0.15	0.54	0.52	0.38		0.28	0.19	0.15	4.01
Subcutaneous injection of *10 cc. 3 per cent calcium lactate 2 hours* previous to epinephrin.										
15	2,000	0.10	0.47	0.46	0.28		0.27	0.23	0.15	4.57
16	1,800	0.15	0.40	0.49	0.29		0.29	0.25	0.18	3.60
24	2,200	0.18	0.51	0.44	0.31	0.28	0.25	0.24	0.19	7.60
25	2,000	0.15	0.46	0.44	0.31	0.30	0.28	0.24	0.12	5.82
Average..		0.15	0.46	0.46	0.30	0.29	0.27	0.24	0.16	5.39
Subcutaneous injection of *10 cc. 3 per cent calcium lactate 3 hours* previous to epinephrin.										
20	2,500	0.17	0.47	0.43	0.53	0.36	0.30	0.19		5.70
21	2,000	0.16	0.44	0.40	0.57	0.37	0.26	0.18		2.87
Average..		0.17	0.46	0.42	0.55	0.37	0.28	0.19		4.28
Subcutaneous injection of *10 cc. 3 per cent calcium lactate 4 hours* previous to epinephrin.										
18	2,200	0.12	0.33	0.31	0.25	0.25	0.18			2.67
19	2,200	0.15	0.25	0.39	0.40	0.27	0.17			3.04
Average..		0.14	0.29	0.35	0.33	0.26	0.18			2.85

Rabbits 8, 9, and 10, Chart 3, the effect of a 1.0 per cent solution of calcium chloride given ½ hour previous to epinephrin may be observed. The amount of sugar appearing in the urine has been markedly increased. Chart 3 shows that calcium chloride injected under the cited conditions has left unchanged the general character of the epinephrin hyperglycemia curve.

When the same dose of calcium lactate was introduced 2 hours previous to epinephrin, glycosuria was even more marked (Table III, Rabbits 13 and 14) and the curve of hyperglycemia was

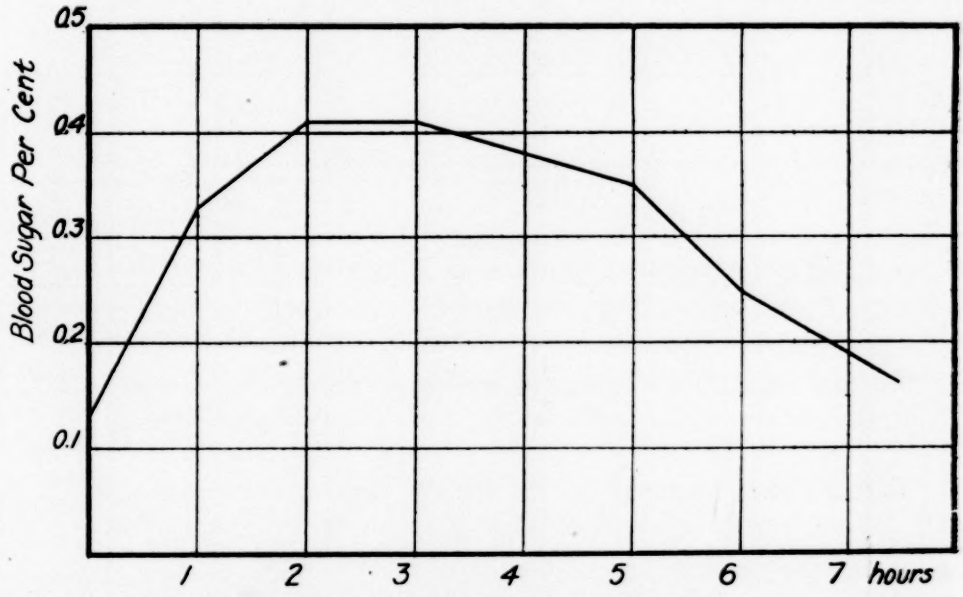

CHART 3. Curve showing the influence of the subcutaneous injection of 10 cc. of 1.0 per cent calcium chloride solution upon epinephrin hyperglycemia. Calcium was injected ½ *hour* previous to epinephrin. Average of three experiments (Table III, Rabbits 8, 9, and 10).

radically changed (Chart 4), the maximum being attained sooner; that is, between the 1st and 2nd hours instead of between the 3rd and 5th hours. Injection of a larger quantity of calcium lactate 2 hours previous to epinephrin administration caused a still greater average output of sugar (Table III, Rabbits 15, 16, 24, and 25), the general character of the hyperglycemia curve (Chart 5) remaining comparable to that of Chart 4. Apparently the injection of 10 cc. of 3.0 per cent calcium lactate 2 hours previous to epinephrin treatment represents the optimum conditions under

the limitations of this investigation, for after the subcutaneous introduction of 10 cc. of a 3.0 per cent solution of calcium lactate 3 hours previous to epinephrin injection the hyperglycemia curve (Chart 6) shows a tendency to return to that of the standard epinephrin curve, the maximum elevation occurring between the 3rd and 4th hours. Likewise the average quantity of sugar excreted is diminished (Table III, Rabbits 20 and 21). This

CHART 4. Curve showing the influence of the subcutaneous injection of 10 cc. of 1.0 per cent calcium lactate solution upon epinephrin hyperglycemia. Calcium was injected *2 hours* previous to epinephrin. Average of two experiments (Table III, Rabbits 13 and 14).

return to the standard epinephrin curve is still further emphasized by an inspection of Chart 7 which shows the influence of 10 cc. of a 3.0 per cent solution of calcium lactate injected 4 hours previous to epinephrin treatment. The average sugar output is also still further diminished (Table III, Rabbits 18 and 19).

These results make it apparent that calcium salts, although devoid of any measurable influence upon blood sugar content

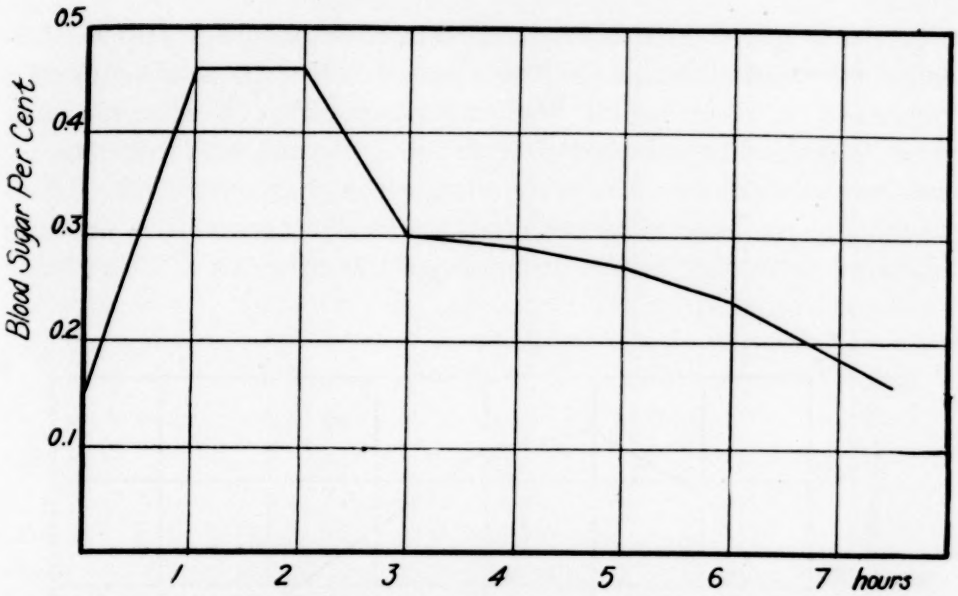

CHART 5. Curve showing the influence of the subcutaneous injection of 10 cc. of 3.0 per cent calcium lactate solution upon epinephrin hyperglycemia. Calcium was injected *2 hours* previous to epinephrin. Average of four experiments (Table III, Rabbits 15, 16, 24, and 25).

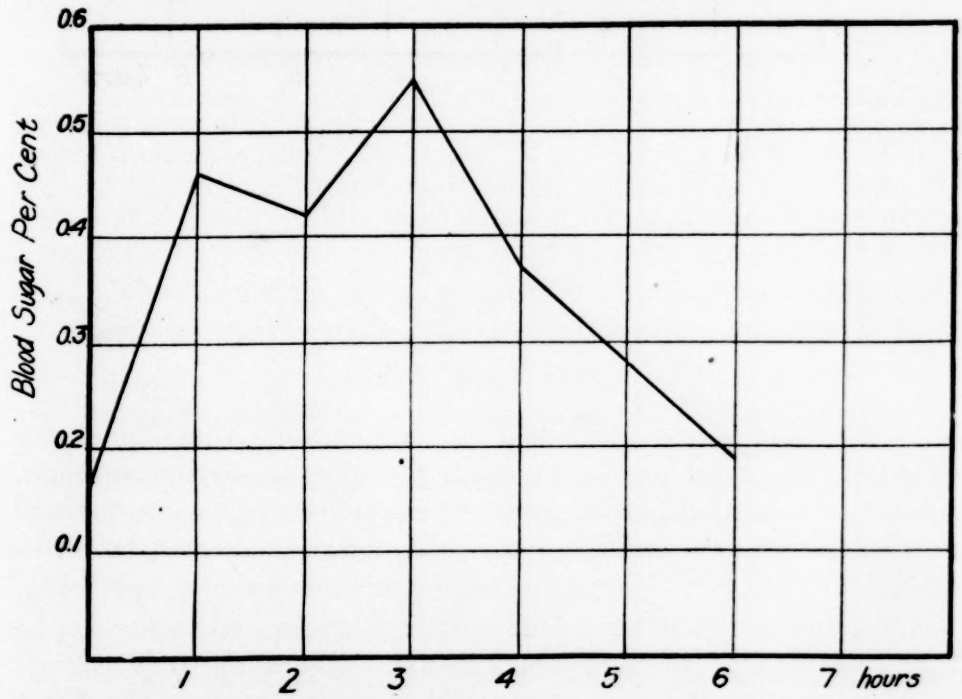

CHART 6. Curve showing the influence of the subcutaneous injection of 10 cc. of 3.0 per cent calcium lactate solution upon epinephrin hyperglycemia. Calcium was injected *3 hours* previous to epinephrin. Average of two experiments (Table III, Rabbits 20 and 21).

when introduced into normal rabbits, may show an appreciable effect when administered suitably to animals with a subsequently disturbed equilibrium of the sugar-regulating mechanism. It may therefore be concluded that the subcutaneous injection of calcium lactate may distinctly change the character of the curve of epinephrin hyperglycemia and markedly increase the amount of sugar appearing in the urine under the stimulus of this glycosuria-producing drug.

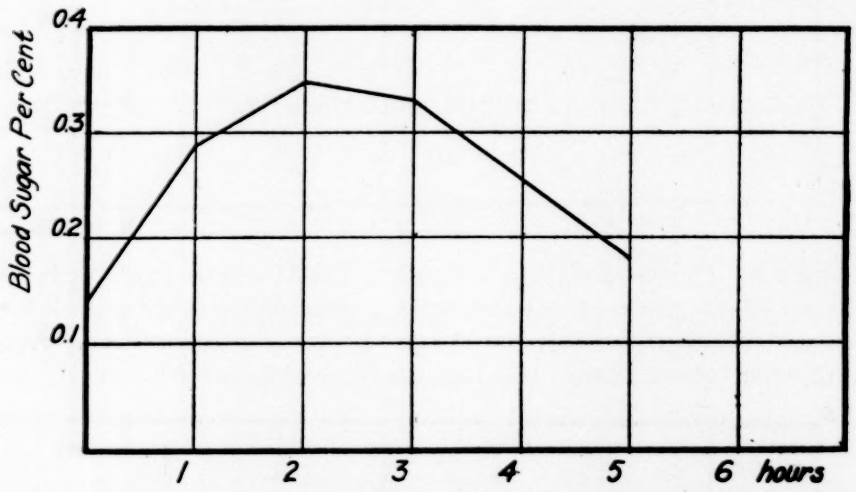

CHART 7. Curve showing the influence of the subcutaneous injection of 10 cc. of 3.0 per cent calcium lactate solution upon epinephrin hyperglycemia. Calcium was injected *4 hours* previous to epinephrin. Average of two experiments (Table III, Rabbits 18 and 19).

The Influence of Sodium Phosphate and of Sodium Oxalate upon Blood Sugar Content and upon Epinephrin Hyperglycemia and Glycosuria.

In a recent paper Starkenstein[12] has claimed that the in roduction of orthophosphoric, metaphosphoric, pyrophosphoric, phytic (inosite-phosphoric), and citric acids when administered as sodium salts to animals, by mouth, subcutaneously, or intravenously, calls forth symptoms which include muscle twitching, tremor, and convulsions. This influence is ascribed to their common function as calcium precipitants. The symptoms evoked in this manner may be relieved by calcium administrations.

[12] Starkenstein, E., *Arch. exp. Path. u. Pharm.*, 1914, lxxvii, 45.

Greenwald[13] has repeated some of these experiments and has reported his failure to obtain the effects mentioned by Starkenstein.[14]

Leaving out of consideration the question whether these sodium salts cause tetany symptoms, it may be accepted that their introduction into the organism will undoubtedly act to rob it of calcium. If calcium injections will increase epinephrin glycosuria and will alter the character of the curve of epinephrin hyperglycemia, what effect upon blood sugar content may be expected when calcium is withdrawn from the normal organism? Or, if there is no appreciable change in blood sugar content, will the withdrawal of calcium change the features of epinephrin hyperglycemia and glycosuria? The results obtained by subcutaneous and intravenous administration of sodium phosphate upon the blood sugar content of normal rabbits may be seen in Table IV. Whichever method of introduction is employed it is evident that blood sugar content may be appreciably lowered temporarily. This influence is not invariable, however, for some animals fail to respond; for example, Rabbit 105. With intravenous injections the effect appears sooner and is more transient than with subcutaneous administration. It is possible, therefore, that withdrawal of calcium from the body will cause a distinct lowering of the sugar in the blood. However, before ascribing this influence to calcium withdrawal a second factor must be considered; namely, the alkalinity of the phosphate solution. To determine whether the diminished blood sugar content is due to the withdrawal of calcium or to the introduction of alkali, experiments were carried through with a neutral calcium-precipi-

[13] Greenwald, I., *J. Pharm. and Exp. Therap.*, 1915, vii, 57.

[14] In my hands the subcutaneous injection of sodium phosphate (Na_3PO_4) in the doses employed in this investigation never produced any symptoms whatsoever. If intravenous introduction was made too rapidly, respiratory difficulties were in evidence. On the other hand, even though no symptoms were apparent during the period of injection, striking effects were seen about $\frac{3}{4}$ of an hour later. These consisted of peculiar head movements, the nose being held high in the air. These motions remind one strongly of those sometimes seen in dogs in incipient tetany after thyreoparathyroidectomy; they last for about 1 hour and then gradually cease. But even for an hour or more after the head has been held quiet the movements may be initiated again for a brief period by disturbing the animal, picking it up, etc.

tating salt, sodium oxalate (Table V). In this instance the initial action is definitely to increase the sugar of the blood, which may later, in certain experiments at least, fall below normal. It is

TABLE IV.

The Influence of Subcutaneous and Intravenous Injections of Sodium Phosphate upon Blood Sugar Content.

Rabbit.	Body weight.	Blood sugar content (in percentages).								Remarks.
		Normal.	Hours after phosphate injection.							
			1	2	3	4	5	6	7	
	gm.									Subcutaneous injections.
38	2,000	0.16	0.29	0.12	0.07	0.06	0.02	0.10	0.11	10 cc. 0.5 N Na_3PO_4.
39	3,000	0.17	0.22	0.09	0.06	0.04	0.10	0.12	0.11	15 " 0.5 " "
41	2,800	0.15	0.15	0.14	0.12	0.02	0.03	0.08	0.10	15 " 0.5 " "
104	2,800	0.16	0.12	0.08	0.09	0.12	0.13			20 " 0.5 " "
105	1,800	0.14	0.15	0.11	0.11	0.14	0.12			10 " 0.5 " "
										Intravenous injections.
96	2,400	0.14	0.10	0.04	0.02	0.13	0.14			100 cc. 0.1 M Na_3PO_4 in
97	2,800	0.14	0.16	0.05	0.05	0.14	0.12			4 to 6 minutes in each
100	2,000	0.16	0.16	0.04	0.08	0.10				instance.
101	2,000	0.16	0.15	0.08	0.16	0.17				

TABLE V.

The Influence of Subcutaneous Injections of Sodium Oxalate upon Blood Sugar Content.

Rabbit.	Body weight.	Blood sugar content (in percentages).										Sugar in urine.	Injection of 0.5 N sodium oxalate.
		Normal.	Hours after oxalate injection.										
			1	2	3	4	5	6	7	8	9		
	gm.											*gm.*	*cc.*
28	2,000	0.18	0.23	0.29	0.31		0.17		0.04	0.07	0.06	Present.	10
29	2,200	0.18	0.23	0.20	0.15		0.13		0.08	0.03		Absent.	20
30	2,700	0.15	0.22	0.25	0.17	0.17	0.18		0.15	0.03	0.03	0.08	10
31	2,300	0.14	0.27	0.30	0.23	0.23	0.23		0.18	0.02	0.04	0.20	10
32	2,200	0.16		0.19		0.16		0.10		0.10		Absent.	10
33	2,000	0.16		0.23		0.17		0.11		0.10		"	10
34	2,000	0.17	0.20		0.14		0.11	0.12	0.14	0.15	0.17	"	10
35	2,000	0.17		0.30		0.19		0.17	0.16	0.23	0.27	Present.	15
36	2,200	0.15		0.18		0.14		0.03	0.04	0.06		0.07	10
37	2,200	0.17		0.22		0.17		0.17	0.10	0.13	0.17	Absent.	10

evident, therefore, that the apparent withdrawal of calcium will cause a fall in blood sugar content. Parenthetically it may be stated that alkali will produce the same result, and a discussion of the possibilities as to the manner in which these facts may be correlated will be discussed in a later paper.

In order to demonstrate even more conclusively the influence that calcium withdrawal may exert upon blood sugar changes, experiments were so devised that sodium phosphate solutions

TABLE VI.

The Influence of Subcutaneous Injections of Sodium Phosphate upon the Hyperglycemia and Glycosuria Provoked by Epinephrin.

Rabbit.	Body weight.	Blood sugar content (in percentages).										Sugar in urine.	Injection of 0.5 N sodium phosphate.
		Normal.	Hours after injection of										
			Sodium phosphate.				Epinephrin.						
			1	2	3	4	1	2	3	4	5		
	gm.											*gm.*	*cc.*
48	2,100	0.16	0.16				0.20	0.34	0.29	0.21	0.15	2.69	10
49	2,800	0.15	0.23				0.26	0.40	0.31	0.27	0.16	5.24	15
Average..		0.15					0.23	0.37	0.30	0.24	0.15	3.96	
46	2,400	0.11	0.12	0.13			0.38	0.30	0.28	0.19	0.16	0.99	10
47	2,000	0.14	0.13	0.13			0.28	0.28	0.26	0.21	0.19	5.01	10
Average..		0.13					0.33	0.29	0.27	0.20	0.17	3.00	
43	2,000	0.18	0.15	0.15	0.14	0.05	0.23	0.28	0.33	0.25	0.17	1.64	10
45	2,000	0.17	0.16	0.16	0.18		0.24	0.30	0.30	0.25	0.18	1.19	10
50	2,500	0.15	0.13	0.14	0.06	0.04	0.12	0.29	0.22	0.16		0.67	10
Average..		0.16					0.19	0.29	0.28	0.22	0.17	1.16	

were injected at varying periods prior to epinephrin administration. The results are to be found in Table VI. It will be observed that when phosphate is introduced 1 hour previous to epinephrin injection (Experiments 48 and 49) there is a less marked degree of hyperglycemia than in the standard epinephrin curve. Moreover, the normal is regained more rapidly. In spite of these facts the total sugar output was higher than when epinephrin was given alone (compare with Table I). Epinephrin injected 2 hours after administration of sodium phosphate (Experi-

ments 46 and 47) yielded the same type of curve as those of Experiments 48 and 49. Here the total output of sugar was below the standard epinephrin excretion in one instance and much above it in the other. If 3 or more hours are allowed to elapse between the introduction of sodium phosphate and that of epinephrin (Experiments 43, 45, and 50) the blood sugar curve is much less elevated and prolonged than that provoked by epinephrin alone, and the average sugar excretion is much below the standard output induced by epinephrin. It is therefore apparent that injections of sodium phosphate will markedly diminish and shorten the period of hyperglycemia caused by epinephrin and will also appreciably decrease the elimination of sugar in the urine.

SUMMARY.

Under the experimental conditions subcutaneous injections of calcium chloride and calcium lactate fail to induce significant changes in the blood sugar content of normal rabbits.

Subcutaneous administration of calcium salts previous to epinephrin introduction may provoke distinct alterations in the character of the curve of epinephrin hyperglycemia and always increase the output of urinary sugar. The time relation between the injection of calcium and of epinephrin and the quantity of calcium salt given constitute the factors which determine the degree of deviation which the curve of blood sugar content may assume. In general, the most marked effect is obtained, both upon the epinephrin hyperglycemia curve and the excretion of sugar, if 10 cc. of 1 to 3 per cent calcium lactate solution are introduced 2 hours previous to the injection of epinephrin.

The subcutaneous or intravenous injection of trisodium phosphate may cause, although not invariably, a distinct diminution in the blood sugar content of normal rabbits. The subcutaneous administration of sodium oxalate may call forth a similar influence, although the condition may be first preceded by an augmented blood sugar content. It is probable that this action of these salts is attributable to their common property of combining with calcium.

Given subcutaneously to rabbits under suitable conditions trisodium phosphate will cause a marked lowering and shortening

of the curve of epinephrin hyperglycemia and the excretion of sugar in the urine may be decreased significantly.

CONCLUSIONS.

Calcium salts play a noteworthy rôle in the regulation of blood sugar content, for (1) although an increase of calcium is without marked effect in normal animals such an augmentation in rabbits with a disturbed carbohydrate metabolism, in epinephrin hyperglycemia, may result in a distinct change in the character of the curve of epinephrin hyperglycemia and may also cause a noticeably increased elimination of sugar in the urine; (2) withdrawal of calcium by introduction of sodium phosphate, for example, will produce in normal rabbits, although not invariably, a condition of hypoglycemia; and (3) the injection of epinephrin at an optimum period after sodium phosphate introduction produces a briefer period of hyperglycemia with a lower sugar level than that yielded by epinephrin alone. The sugar elimination by the kidney may also be less than the average output under epinephrin only.

STUDIES IN CARBOHYDRATE METABOLISM.

XII. THE INFLUENCE OF SODIUM CARBONATE UPON BLOOD SUGAR CONTENT AND UPON EPINEPHRIN HYPERGLYCEMIA AND GLYCOSURIA.*

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Under normal conditions the blood sugar content may be accepted as constituting one of the body constants. The exact mechanism whereby this regulation is maintained is unknown. The introduction into the organism of various types of substances, such as epinephrin, phlorhizin, phosphorus, hydrazine, etc., is succeeded by a temporary failure of the blood sugar-regulating apparatus which may be manifested by the development of hyperglycemia with glycosuria or by a condition of hypoglycemia in which sugar may or may not appear in the urine. The equilibrium of the blood sugar-regulating mechanism is undoubtedly exceedingly sensitive to a great variety of chemical influences. This is particularly true with respect to circumstances which tend toward the production of a condition of acidosis, as exemplified by the hyperglycemia and glycosuria which may be evoked by dyspnea and allied states. That acidosis exerts a distinct influence upon carbohydrate metabolism is attested by the observations of Elias,[1] who demonstrated that the introduction of hydrochloric acid into dogs and rabbits leads to hyperglycemia and glycosuria. Elias[2] has also shown that the so called hunger diabetes[3] of young dogs is, in part at least, a condition due to

* Reported at the December meeting of the American Society for Biological Chemists, Boston, 1915. See also *Proc. Soc. Exp. Biol. and Med.*, 1916, xiii, 111.

[1] Elias, H., *Biochem. Z.*, 1912–13, xlviii, 120.

[2] Elias, H., and Kolb, L., *Biochem. Z.*, 1913, lii, 331.

[3] Hofmeister, F., *Arch. exp. Path. u. Pharm.*, 1889–90, xxvi, 355.

acidosis, as indicated by the carbon dioxide content of the blood and analysis of the alveolar air. Observations upon human diabetes also teach that in this condition acidosis may play a part. From this brief review it is evident that the introduction of acid or the production of acid in the organism is associated with a disturbance of blood sugar content and is manifested in one direction; namely, blood sugar content is augmented. On the other hand, the influence of a state of alkalosis upon blood sugar content has received little or no attention.

While attempting to demonstrate the relation of calcium to blood sugar content, as reported in the preceding paper, it was observed that the introduction of alkali into the blood stream sometimes caused a marked diminution in the sugar of the blood. In the present communication are given the results of these experiments together with those designed to test the influence of alkali upon epinephrin hyperglycemia and glycosuria.

Methods.

The general methods employed were those outlined in the preceding article. Well fed, full grown rabbits received into the ear vein injections of sodium carbonate solutions, the period of introduction varying from 2 to 5 minutes. Anesthesia was never resorted to. Usually the only symptom observable was a greatly diminished respiration and noticeable peristalsis. Blood for analysis was withdrawn from the ear vein, sugar being estimated by the Forschbach and Severin method. Adrenalin (Parke, Davis and Company 1 : 1,000) was introduced subcutaneously in the dosage of 1 cc. per kilo of body weight. Sugar in the urine was estimated by the Allihn procedure.

The Influence of Intravenous Injections of Sodium Carbonate upon Blood Sugar Content.

That alkali introduction exerts an action upon carbohydrate regulation in the body was demonstrated by Pavy and Godden,[4] who found that the glycosuria provoked by ether or chloroform disappears after the intravenous injection of sodium carbonate. Given by mouth or intravenously sodium carbonate will abolish the hyperglycemia of hunger diabetes, and the glycosuria, according to Elias,[2] will either entirely disappear or be greatly dimin-

[4] Pavy, F. W., and Godden, W., *J. Physiol.*, 1911–12, xliii, p. vii.

ished. After removal of the pancreas, sodium carbonate introduced into the blood stream of the dog causes a diminution of the sugar excretion.[5] Later work by Murlin and Kramer[6] has shown that under the influence of sodium hydroxide the respiratory quotient is increased in depancreatized dogs. In agreement with this finding are the results of Kramer and Marker[7] who have stated that, "Sugar retained when sodium carbonate is administered to depancreatized dogs is not stored as glycogen nor is it eliminated by way of the saliva or the gastro-intestinal tract." These observations make it evident that a condition of alkalosis may exert an influence upon carbohydrate metabolism, but this effect is manifested by retention of carbohydrate within the organism.

The explanation for the closely agreeing observations just cited probably lies in the effect which sodium carbonate exerts at times upon blood sugar content. This influence may be seen by reference to Table I. It is evident from these figures that the intravenous administration of sodium carbonate will cause a marked diminution in the blood sugar content of normal rabbits, but that this effect is not invariable. It will be observed that the quantity of alkali injected bears little relation to the action exerted upon blood sugar content. Expressed differently, one may say that the introduction of approximately 0.5 gm. of sodium carbonate is just as efficacious when dissolved in 100 cc. of water as it is in stronger concentration or even in greater absolute amount. Blood sugar content is not always[8] affected but when there is a diminution it is transient, the normal being regained usually within 1 or 1½ hours. If a second injection is given at a period when blood sugar is still low, the interval of hypoglycemia may be lengthened noticeably (Rabbit 92). The diminution in the percentage of blood sugar cannot be ascribed to mere dilution of the blood by the introduced fluid, because it has been found by experiments in this laboratory[9] that under the conditions

[5] Murlin, J. R., and Kramer, B., *J. Biol. Chem.*, 1913, xv, 365.

[6] Murlin and Kramer, *J. Biol. Chem.*, 1916, xxiv, p. xxv.

[7] Kramer, B., and Marker, J., *J. Biol. Chem.*, 1916, xxiv, p. xxiv.

[8] The possible reasons for the irregularities in this respect are being investigated.

[9] To be published shortly.

TABLE I.

The Influence of Intravenous Injections of Sodium Carbonate upon Blood Sugar Content.

Rabbit.	Body weight.	Blood sugar content (in percentages).							Remarks.
		Normal.	Hours after carbonate injection.						
			½	1	1½	2	3	4	
	gm.								Injection of 0.4 per cent Na_2CO_3.
82	2,500	0.15		0.05		0.11	0.09	0.11	100 cc.
83	2,000	0.14		0.05		0.12	0.13	0.11	80 "
84	2,100	0.12	0.05	0.04	0.10	0.09			10.20 a.m., 100 cc.
		0.15	0.03	0.03	0.02	0.11			2.30 p.m., 100 "
85	2,000	0.12	0.02	0.05	0.12	0.15			10.20 a.m., 100 "
		0.15	0.03	0.03	0.02	0.11			2.30 p.m., 100 "
94	2,000	0.17	0.16	0.11					100 cc.
95	2,000	0.16	0.12	0.09					100 "
99	2,000	0.18	0.13	0.12		0.17	0.15		120 "
									Injection of 1.0 per cent Na_2CO_3.
102	1,600	0.11	0.18	0.15		0.18	0.16		10.30 a.m., 80 cc.
		0.16	0.14	0.13		0.12			2.30 p.m., 80 "
103	1,600	0.16	0.14	0.13		0.12	0.12		10.30 a.m., 80 "
		0.12	0.12	0.13		0.14			2.30 p.m., 80 "
									Injection of 2.0 per cent Na_2CO_3.
92	1,600	0.13	0.05	0.04	0.04	0.05	0.04	0.14	10.30 a.m., 30 cc.
									11.30 " 20 "
93	1,600	0.13	0.02	0.05					30 cc. Killed by gas bubble during second injection.

of the present experiments blood volume is restored to normal within a period of 20 to 25 minutes.

The regulation of blood sugar content would appear to be associated in part at least with the equilibrium between acids and bases in the body. Acid tends to increase blood sugar content; it has an influence in causing sugar elimination. On the other hand, base or alkali tends to reduce the blood sugar content. These ideas fit in well with the facts known concerning the relation of thyreoparathyroidectomy to sugar metabolism. It has been shown by Underhill and Blatherwick[10] that previous

[10] Underhill, F. P., and Blatherwick, N. R., *J. Biol. Chem.*, 1914, xviii, 87; xix, 119.

to the tetany provoked by removal of the thyroid-parathyroid mechanism, blood sugar content may be greatly diminished. Calcium relieves the tetany and restores blood sugar to normal. In the preceding paper of this series it has been demonstrated that under disturbed conditions of carbohydrate metabolism calcium behaves in a manner analogous to hydrochloric acid. Wilson and his coworkers[11] have found that previous to the onset of tetany after removal of the thyroids and parathyroids a condition of alkalosis obtains. The introduction of hydrochloric acid relieves the symptoms equally as well as calcium administration. The period of alkalosis corresponds fairly well with the interval of low blood sugar observed by Underhill and Blatherwick. It is probable, therefore, that the condition of alkalosis may be responsible for the hypoglycemia. The resumption of normal blood sugar is induced by either calcium or hydrochloric acid which have a common behavior in increasing blood sugar content under suitable conditions.

The Effect of Intravenous Injections of Sodium Carbonate upon Epinephrin Hyperglycemia and Glycosuria.

Both calcium and hydrochloric acid cause a marked augmentation of epinephrin hyperglycemia and glycosuria. In view of the fact that sodium carbonate may bring about a condition of hypoglycemia in normal rabbits and decrease the hyperglycemia in dogs after pancreas removal, it seemed desirable to determine the action of sodium carbonate upon the hyperglycemia and glycosuria induced by epinephrin. This has been done and the results obtained are detailed in Table II.

When sodium carbonate is introduced into the blood stream in quantities above 0.5 gm. at an interval of ½ to 1 hour previous to epinephrin administration, there is a marked influence upon both the hyperglycemia and glycosuria. The hyperglycemia is distinctly lower and its period of duration decidedly shorter than when the sodium carbonate has been omitted.[12] Likewise the

[11] Wilson, D. W., Stearns, T., and Thurlow, M. D., *J. Biol. Chem.*, 1915, xxiii, 100. Wilson, D. W., Stearns, T., and Janney, J. H., Jr., *ibid.*, 1915, xxiii, 123.

[12] See preceding paper for hyperglycemia curve.

TABLE II.

The Influence of Intravenous Injections of Sodium Carbonate upon Epinephrin Hyperglycemia and Glycosuria.

Rabbit.	Body weight.	Blood sugar content (in percentages).														Sugar in urine.	Remarks.
		Normal.	Hours after injection of														
			Sodium carbonate.		Epinephrin.												
			½	1	½	1	1½	2	2½	3	3½	4	4½	5	5½		
	gm.															*gm.*	
																	Injection of 0.4 per cent Na_2CO_3.
86	2,400	0.12	0.02	0.04		0.11		0.23		0.18			0.15			0.54	100 cc. + epinephrin.
						0.26		0.27								0.72	Epinephrin alone 5 hours after first injection.
87	1,800	0.12	0.02	0.05		0.15		0.15		0.12			0.16			0.00	80 cc. + epinephrin.
						0.27		0.24								0.83	Epinephrin alone 5 hours after first injection.
																	Injection of 2.0 per cent Na_2CO_3.
90	1,800	0.12	0.13		0.13	0.13		0.18		0.30		0.07	0.06			0.20	30 cc.
91	2,200	0.11	0.12		0.11	0.26		0.26		0.24		0.19	0.15			0.75	35 "
																	Injection of 1.0 per cent Na_2CO_3.
106	2,200	0.15	0.12			0.21	0.30		0.32		0.31		0.24		0.13	2.24	75 cc.
107	2,200	0.14	0.10			0.15	0.23		0.26		0.24		0.15		0.14	0.76	100 "
108	2,200	0.13	0.13			0.10	0.16	0.26	0.29		0.25		0.25		0.19	0.48	125 "
109	2,100	0.13	0.15			0.16	0.16	0.21	0.26		0.29		0.30		0.12	0.26	125 "
																	Injection of 0.4 per cent Na_2CO_3 and epinephrin simultaneously.
88	1,600	0.11			0.27	0.27		0.40		0.20		0.16		0.13		2.43	75 cc.
89	2,000	0.12			0.19	0.29		0.26		0.23		0.16		0.17		2.40	100 "

sugar elimination in general falls far below the average excretion observed when only epinephrin has been injected. In one instance (Rabbit 87) neither increased blood sugar nor glycosuria was in evidence. That this animal was not glycogen-free is proved by the fact that a second injection of epinephrin on the same day called forth an excretion of an appreciable quantity of sugar. Evidently it is not essential that there be an initial drop in blood sugar previous to epinephrin injection in order to obtain the typical influence upon epinephrin hyperglycemia and glycosuria. This may be seen readily from Rabbits 90 to 109 inclusive. It is essential, however, that at least $\frac{1}{2}$ hour should elapse between the administration of sodium carbonate and that of epinephrin, as may be seen from the results with Rabbits 88 and 89 where the sodium carbonate and epinephrin were given nearly simultaneously. The extent and duration of hyperglycemia[12] more nearly resemble those observed with epinephrin alone. Even in this case, however, the duration of hyperglycemia has been significantly decreased. Glycosuria was also very much greater in both cases than in any of those, with a single exception, where an appreciable time interval occurred between the two injections.

CONCLUSIONS.

The intravenous injections of sodium carbonate into rabbits may induce a marked though transient fall in blood sugar content.

It is suggested that the acid-base equilibrium is a factor in blood sugar regulation.

The significance of this view in its relation to carbohydrate metabolism after thyreoparathyroidectomy is indicated.

The hyperglycemia and glycosuria provoked by epinephrin are both significantly decreased if sodium carbonate is administered at suitable periods of time previous to epinephrin introduction.

STUDIES IN CARBOHYDRATE METABOLISM.

XIII. THE INFLUENCE OF MAGNESIUM SALTS UPON BLOOD SUGAR CONTENT AND UPON EPINEPHRIN HYPERGLYCEMIA AND GLYCOSURIA.

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In a previous communication[1] it has been pointed out that under suitable conditions calcium may exert a significant influence upon certain phases of carbohydrate metabolism. Since it is generally assumed that calcium and magnesium within the organism may play antagonistic rôles it seemed desirable to investigate more closely than has been done previously the relationship of magnesium to blood sugar content both under normal conditions and in hyperglycemia experimentally produced. Our knowledge of the possible influence of magnesium upon carbohydrate metabolism is slight. Meltzer and Auer,[2] during the course of an investigation on the general behavior of magnesium salts in the body, incidentally observed the appearance of sugar in the urine. Underhill and Closson[3] who studied the phenomenon more closely showed that the intravenous injection of magnesium sulfate caused a significant hyperglycemia which they ascribed to disturbances of respiration, dyspnea, induced by the administration of magnesium. A recent report[4] by Kleiner and Meltzer confirms these results, although the latter investigators believe that the condition of hyperglycemia is evoked in some specific manner by the magnesium salt. Airila and Bardy[5] state

[1] Underhill, F. P., *J. Biol. Chem.*, 1916, xxv, 447.
[2] Meltzer, S. J., and Auer, J., *Am. J. Physiol.*, 1905–06, xiv, 371.
[3] Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1905–06, xv, 321.
[4] Kleiner, I. S., and Meltzer, S. J., *J. Biol. Chem.*, 1916, xxiv, p. xx.
[5] Airila, Y., and Bardy, H., *Skand. Arch. Physiol.*, 1915, xxxii, 246.

that the intravenous injection of magnesium salts diminishes or inhibits the glycosuria produced by epinephrin.

The present investigation was planned to determine the influence of (*a*) small doses of magnesium upon blood sugar content in normal animals, (*b*) small doses of magnesium upon epinephrin hyperglycemia and glycosuria, (*c*) anesthetic doses of magnesium salts upon blood sugar content and upon epinephrin hyperglycemia and glycosuria, and (*d*) the influence of calcium upon the extent and character of the hyperglycemia and glycosuria provoked by magnesium salts.

Methods.

Rabbits were employed throughout. Estimation of sugar in the blood and urine was made in accordance with the procedures cited in a previous paper.[1] Magnesium was injected subcutaneously and calcium was introduced through the marginal ear vein. The epinephrin used was adrenalin of Parke, Davis and Company 1 : 1,000, 1 cc. or 1 mg. per kilo of body weight constituting the dosage employed.

The Influence of Subcutaneous Injections of Magnesium Lactate upon Blood Sugar Content and upon Epinephrin Hyperglycemia and Glycosuria.

It may be seen from an inspection of Table I that in the doses employed a 3 per cent solution of magnesium is without influence upon the blood sugar content of normal animals during a period of 6 hours. The introduction of magnesium under the experimental conditions is attended by no symptoms.

TABLE I.

The Influence of Magnesium Lactate upon Blood Sugar Content.

Rabbit.	Body weight.	Blood sugar content (in percentages).							Subcutaneous injection of 3 per cent solution of magnesium lactate.
		Normal.	Hours after injection of magnesium lactate.						
			1	2	3	4	5	6	
	gm.								*cc.*
54	2,000	0.16	0.15	0.14	0.13	0.11	0.12	0.10	10
55	2,000	0.13	0.15	0.12	0.14	0.14	0.10	0.10	10
56	2,000	0.13	0.15	0.16	0.13	0.12	0.13	0.15	20
57	2,000	0.11	0.11	0.12	0.14	0.11	0.14	0.12	20

On the other hand, if comparable doses of magnesium are given to rabbits at various periods previous to epinephrin administration a distinct influence may be observed both upon the curve of hyperglycemia and upon the sugar elimination by the kidney (Table II). The effect upon hyperglycemia is manifested chiefly by a significant shortening of the duration of this condition, the maximum of the hyperglycemia not being influenced appreciably. The maximum effect upon hyperglycemia appears to be produced when the magnesium salt has been introduced 1 or 2 hours previous to the injection of epinephrin. A distinctly lower level and shorter duration of hyperglycemia are to be noted when the epinephrin has been administered 3 hours after the introduction of magnesium. Magnesium lac-

TABLE II.

The Influence of Magnesium Lactate upon Epinephrin Hyperglycemia and Glycosuria.

Rabbit.	Body weight.	Blood sugar content (in percentages).										Sugar in urine.	Subcutaneous injection of 3 per cent solution of magnesium lactate.
		Normal.	Hours after injection of										
			Magnesium lactate.			Epinephrin.							
			1	2	3	1	2	3	4	5	6		
	gm.											*gm.*	*cc.*
58	2,000	0.14	0.13	0.12		0.34	0.34	0.40	0.39	0.28		3.58	
59	2,000	0.14	0.14	0.14	0.14	0.33	0.40	0.35	0.30	0.25		7.72	20
60	2,000	0.18	0.13	0.14		0.33	0.47	0.42	0.30	0.23	0.14	2.85	
61	1,800	0.17	0.11	0.13		0.32	0.50	0.41	0.30	0.29	0.09	2.52	
Average.						0.33	0.43	0.39	0.32	0.26	0.11	4.17	
62	1,800	0.14	0.15			0.37	0.41	0.36	0.21	0.19	0.15	2.96	20
63	3,000	0.13	0.12			0.39	0.39	0.27	0.24	0.20	0.12	3.60	30
Average.						0.38	0.40	0.32	0.23	0.20	0.14	3.28	
64	2,200	0.18	0.17	0.18	0.19	0.33	0.33	0.23	0.23	0.18		2.48	30
65	1,800	0.16	0.17	0.17	0.17	0.21	0.33	0.25	0.22	0.17		1.05	20
66	1,800	0.15			0.17	0.25	0.33	0.32	0.21	0.17		3.15	20
67	1,800	0.14			0.14	0.28	0.35	0.34	0.22	0.17		1.70	20
Average.						0.27	0.33	0.28	0.22	0.17		2.09	

tate seems to favor the elimination of sugar by the kidney during epinephrin hyperglycemia, for the average amount of sugar appearing in the urine is greater when magnesium injection precedes epinephrin introduction than it is when epinephrin is given alone.[1] The maximum effect in this respect is seen if magnesium lactate is injected 2 hours previous to epinephrin.

This influence of magnesium is still manifested if the magnesium injection precedes that of epinephrin by 1 hour. Epinephrin introduced 3 hours after magnesium produces a greater average quantity of sugar than is found after epinephrin administration only, but the quantity of sugar excreted is decidedly less than is called forth by the introduction of epinephrin at periods 1 or 2 hours after magnesium.

Under the experimental conditions magnesium lactate appears to exert an influence upon carbohydrate metabolism similar to that evoked by calcium; that is, both induce a greater output of sugar in epinephrin glycosuria.

The Effect of Anesthetic Doses of Magnesium Sulfate upon Blood Sugar Content and upon Epinephrin Hyperglycemia and Glycosuria.

The results outlined in Table III make it evident that doses of magnesium sulfate too small to produce anesthesia have only a slight transitory influence upon blood sugar content in normal rabbits. This slight effect is in the direction of inducing hyperglycemia, but the degree of high blood sugar content is insufficient to elicit the appearance of sugar in the urine. When, however, magnesium sulfate is injected in quantities leading to anesthesia hyperglycemia is marked and may continue for several hours. Under these circumstances the urine volume is low and the total quantity of sugar excreted is very small.

The effect of anesthetic doses of magnesium sulfate upon epinephrin hyperglycemia and glycosuria may be seen in the experiments detailed in Table IV. It is obvious that under the experimental conditions magnesium does not greatly alter the height or duration of epinephrin hyperglycemia nor is the output of sugar through the urine profoundly changed from that induced by epinephrin alone.

TABLE III.

The Influence of Anesthetic Doses of Magnesium Sulfate upon Blood Sugar Content.

Rabbit.	Body weight.	Blood sugar content (in percentages).								Sugar in urine.	Subcutaneous injection of 2N $MgSO_4$.
		Normal.	Hours after injection of magnesium sulfate.								
			1	2	3	4	5	6	7		
	gm.									*gm.*	*cc.*
68	2,300	0.17	0.18	0.22	0.20	0.14	0.14	0.13	0.13	0	15*
69	3,800	0.15	0.19	0.18	0.17	0.11	0.12	0.13	0.12	0	20*
72	1,900	0.17	0.25	0.37	0.33	0.33	0.25	0.16	0.18	0.80	25†
73	2,200	0.15	0.31	0.48	0.47	0.35	0.22	0.22	0.16	0.36	30†
74	2,400	0.15	0.29	0.40	0.21	0.15				0.65	25†
75	2,000	0.16	0.19	0.29	0.41	0.31	0.19	0.15		0.34	25†

* No anesthesia.

† Complete anesthesia.

TABLE IV.

The Influence of Anesthetic Doses of Magnesium Sulfate upon Epinephrin Hyperglycemia and Glycosuria.

Rabbit.	Body weight.	Blood sugar content (in percentages).										Sugar in urine.	Remarks.
		Normal.	Hours after injection of										
			Magnesium sulfate.		Epinephrin.								
			1	2	1	2	3	4	5	6			
	gm.											*gm.*	
70	2,800	0.18	0.20	0.28	0.42	0.42	0.37	0.37	0.33	0.23		2.16	Subcutaneous injection of 2N $MgSO_4$, 30 cc. Deep anesthesia in 1 hour.
71	2,200	0.18	0.29	0.31	0.37	0.37	0.36	0.36	0.36	0.34		1.89	

The Influence of Intravenous Injections of Calcium Chloride upon the Hyperglycemia and Glycosuria Provoked by Magnesium Sulfate.

Meltzer and Auer[6] have shown that an animal anesthetized by an injection of magnesium sulfate may be quickly restored to a normal state by the intravenous administration of calcium

[6] Meltzer and Auer, *Am. J. Physiol.*, 1908, xxi, 400.

chloride. Since the hyperglycemia and glycosuria subsequent to magnesium injection are most evident during the period of anesthesia it was of interest to determine what influence would be exerted upon blood sugar content by the recovery from anesthesia through calcium administration. The results of four such experiments may be seen in Table V. In each instance the rabbit

TABLE V.

The Influence of Calcium Chloride upon the Hyperglycemia and Glycosuria Provoked by Magnesium Sulfate.

Rabbit.	Body weight.	Blood sugar content (in percentages).				Sugar in urine.	Remarks.
		Normal.	Hours after injection of				
			Magnesium sulfate.	Calcium chloride.			
			1	1	2		
	gm.						
78	2,400	0.13	0.16	0.26	0.13	0	Subcutaneous injection of 25 cc. 2N $MgSO_4$. Complete anesthesia resulted within an hour. 1 hour after the $MgSO_4$ injection each rabbit received 10 cc. 2.5 per cent solution of $CaCl_2$ intravenously. Within 2 minutes the animal had entirely recovered from the deep anesthesia and was sitting in a normal position.
79	2,400	0.13	0.19	0.22	0.14	Trace.	
80	2,100	0.12	0.19	0.10	0.11	0	
81	2,100	0.14	0.20	0.13	0.10	0	

was in a state of profound anesthesia within 1 hour after magnesium injection. The range of blood sugar content usually obtained with magnesium anesthesia may be found in Table III. Comparison of these figures with those of Table V shows that the introduction of calcium caused a rapid reduction of the hyperglycemia produced by magnesium to normal blood sugar content, this effect being manifested within an hour or two. Glycosuria either entirely failed to develop or else was present to a slight extent only.

Inasmuch as it has been shown that calcium salts and hydrochloric acid produce similar types of influence upon blood sugar content during epinephrin hyperglycemia and glycosuria and since calcium chloride has an antagonistic action to magnesium with respect to anesthesia, it seemed desirable to test the influence of hydrochloric acid upon the state of anesthesia evoked by magnesium sulfate. For this purpose a rabbit of 2,200 gm. body weight received an injection of 30 cc. 2 N magnesium sulfate subcutaneously. Deep anesthesia developed in 45 minutes. Intravenous administration of 20 cc. 0.1 per cent hydrochloric acid solution in 3 minutes caused accelerated respiration and return of the eye reflex temporarily, but the conditions peculiar to magnesium anesthesia were soon resumed. A second rabbit of 2,200 gm. body weight was given a subcutaneous injection of 30 cc. 2 N magnesium sulfate solution which was followed by the usual deep anesthesia. The intravenous injection of 20 cc. 1.0 per cent hydrochloric acid solution caused only an accelerated respiration. The influence of alkali introduction was tested upon a third rabbit weighing 3,000 gm. Anesthesia was induced by the subcutaneous injection of 30 cc. 2 N magnesium sulfate solution. The intravenous administration of 60 cc. 1.0 per cent sodium carbonate solution was without noticeable effect.

It is apparent, therefore, that administration of hydrochloric acid or sodium carbonate is without significant influence upon magnesium anesthesia.

SUMMARY.

Small doses of magnesium lactate introduced subcutaneously fail to produce significant changes in the blood sugar content of the rabbit.

The same dosage of magnesium lactate injected in like manner intensifies the effect of epinephrin upon blood sugar content and glycosuria. The maximum influence is seen when the magnesium salt is given 2 hours previous to epinephrin treatment.

A significant hyperglycemia is produced by subcutaneous administration of magnesium sulfate if general anesthesia develops. Glycosuria is always slight. If anesthesia fails to develop, hyperglycemia is not pronounced and is of short duration. Under these conditions sugar fails to appear in the urine.

Injection of calcium during the height of magnesium anesthesia quickly abolishes this state, as demonstrated by Meltzer and Auer, and in correspondence with this antagonism there is a rapid return of blood sugar content to the normal level. Glycosuria is either absent or is present to a small degree only.

Administration of hydrochloric acid or sodium carbonate is without significant influence upon magnesium anesthesia.

HYDROGEN ELECTRODE POTENTIALS OF PHTHALATE, PHOSPHATE, AND BORATE BUFFER MIXTURES.*

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INTRODUCTION.

At the present stage of its development the colorimetric method of determining hydrogen ion concentration requires the use of a set of comparison solutions whose hydrogen ion concentrations have been accurately determined by hydrogen electrode measurements. Of the various sets which have been proposed the most complete and reliable is that of Sörensen (35, 36). This set may be supplemented by various other mixtures among which should be mentioned the acetic acid-sodium acetate mixtures carefully studied by Walpole (38), cacodylate mixtures (30, 37), and Palitzsch's (33) boric acid-borate mixtures.

The present study will add to the available list the phthalate mixtures which received their first brief treatment by Fels (16).

We have also studied a selection of mixtures which as a set seem to us to possess certain advantages over those in common use. In our selection we have been guided largely by the desire to simplify the technique of preparation and to reduce the number of substances required.

We have considered it advisable to eliminate as far as possible the use of substances which crystallize with water of crystallization. Citric acid used in the Sörensen mixtures crystallizes with water of crystallization, and it effloresces so easily that dehydration or else determination of the water content is necessary. Complete elimination of the water without decomposition of the

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acid is a delicate operation. Furthermore, citric acid sometimes crystallizes anhydrous, a fact which might easily cause confusion. We also have reason to believe that in the preparation of $Na_2HPO_4.2H_2O$ from $Na_2HPO_4.10H_2O$ by Sörensen's method some workers have not appreciated the conditions or else the length of exposure necessary.[1] Our objection to the use of substances which crystallize with water of crystallization applies essentially to a substance we have had to retain—boric acid. Boric acid is ordinarily considered to crystallize without water of crystallization inasmuch as it precipitates with a composition which may be expressed as H_3BO_3; nevertheless, it loses water at a comparatively low temperature and is reported to change to metaboric acid between 80 and 100°. We have had no difficulty, however, in obtaining air-dried boric acid which loses less than 0.1 per cent in weight when held for a week over calcium chloride. If we are to believe Berzelius (3) (1835) and the statements frequently made since his observation, borax, if carelessly handled, might become seriously impregnated with carbonate. The same might be true of disodium phosphate. Considerations such as these have led us to reduce the number of alkaline substances and alkaline stock solutions in our set to one.

The acetic acid in the acetate mixtures is volatile and requires somewhat elaborate purification. The acetate mixtures are furthermore prone to support mold growth, although not to the extent which makes glycocoll so troublesome in standard solutions.

The following is the set of mixtures we propose:

Acid potassium *o*-phthalate	—	hydrochloric acid.		
" " "	—	sodium hydroxide.		
" " phosphate	—	"	"	
Boric acid + KCl	—	"	"	

It will be noted that all the solid substances crystallize beautifully from their aqueous solutions without water of crystallization. All but the boric acid may be dried at 110°C. in an ordinary oven. The boric acid may be dried fairly well in bulk by exposing it to the air, on a dry day, and can be spread finally in thin layers

[1] We have observed a 10 per cent increase in the weight of $Na_2HPO_4.2H_2O$ exposed to a humid atmosphere. This is unlikely to occur under ordinary laboratory conditions but may be serious in humid climates.

in a desiccator over calcium chloride. Only the sodium hydroxide need be protected from the CO_2 of the atmosphere.

The proper preparation of the sodium hydroxide solution may appear to be a difficult matter. Later we shall outline a method of preparation which furnishes a solution sufficiently pure for the present purpose.

Fig. 1, which must be drawn to a larger scale from the data given later before it can be used as is Sörensen's chart (36), will serve to show that the curve of the phthalates overlaps the curve of the phosphates sufficiently to allow the preparation of phthalate and phosphate solutions of the same hydrogen ion concentration. In like manner the phosphate and borate curves overlap. This is important, for it makes it possible to check colorimetrically the consistency of any particular set of these solutions. It will also be seen that the phthalate-HCl mixtures run up into the region which may be covered by HCl-KCl mixtures, and, were these added to the list, the entire range of hydrogen ion concentration of any physiological importance could be covered. Some preliminary measurements of KCl-HCl mixtures at 25° are given in Table VII.

Acid potassium phthalate, as shown by Dodge (14) and confirmed by Hendrixson (19), is an excellent substance for the standardization of alkali solutions. We therefore have within the set a substance which may be relied upon to standardize the sodium hydroxide solution and indirectly the hydrochloric acid solution. The other substances can be weighed accurately.

We shall describe in the following pages the electrometric measurements made, and shall discuss the values of diffusion and calomel electrode potentials used in the calculation of the hydrogen electrode potentials and P_H values of these mixtures.

Apparatus and Methods.

General Procedure.—The hydrogen electrode measurements were made with the chain: Hg, HgCl, KCl (0.1 N) / KCl (saturated) / Solution, Pt, H_2. Instead of measuring the potential of this chain directly, it was measured in two steps. First, the chain Hg, HgCl, KCl (saturated) / Solution, Pt, H_2 was measured. The "saturated calomel electrode" was used as recommended by

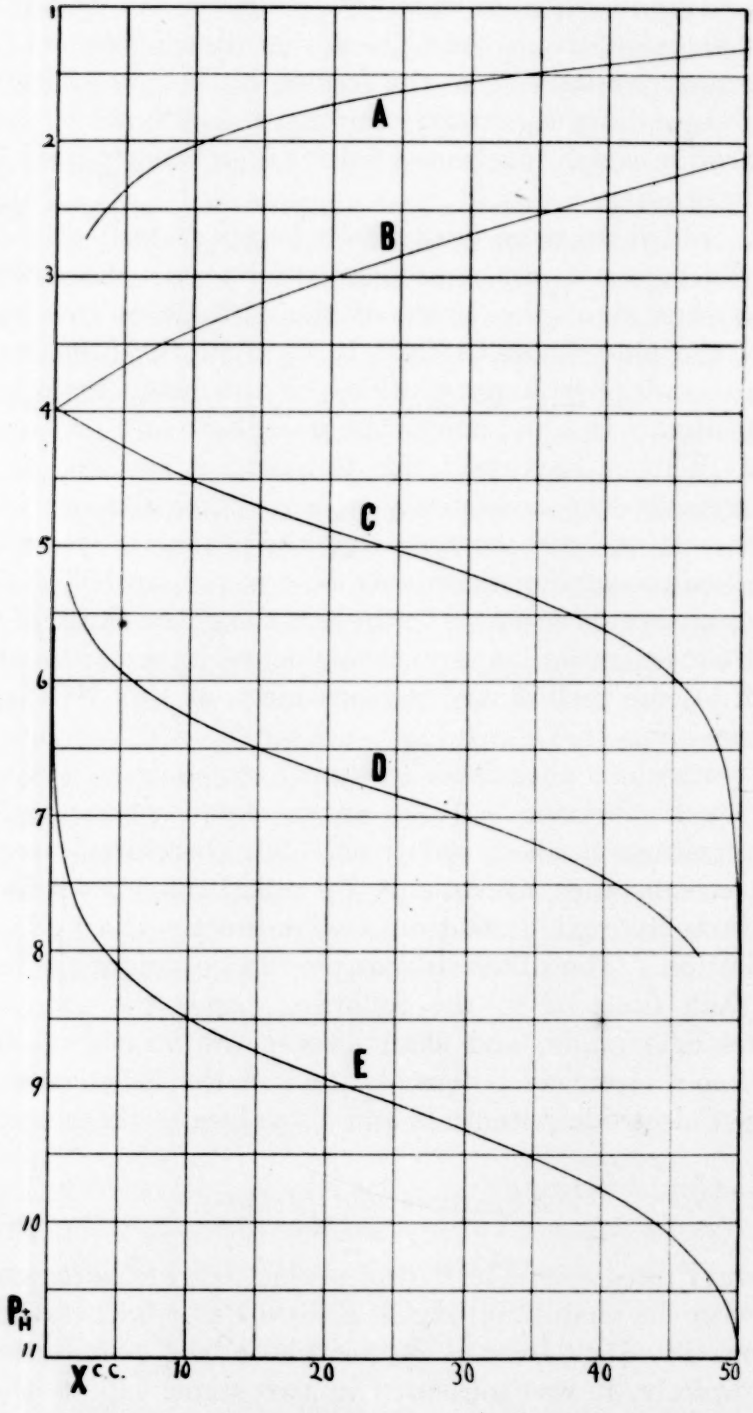

FIG. 1. P_H values of the mixtures:

A. 50 cc. 0.2 M KCl + X cc. 0.2 M HCl. Diluted to 200 cc.

B. 50 " 0.2 " KH Phthalate + X cc. 0.2 M HCl. Diluted to 200 cc.

C. 50 " 0.2 " " + X " 0.2 "NaOH. " " " "

D. 50 " 0.2 " KH_2PO_4 + X cc. 0.2 M NaOH. Diluted to 200 cc.

E. 50 " 0.2 " H_3BO_3, 0.2 M KCl + X cc. 0.2 M NaOH. Diluted to 200 cc.

Michaelis and Davidoff (29), not only because it requires less careful protection from the adjacent concentrated potassium chloride solution, but because its low electrical resistance permits a fuller use of the galvanometer's sensitivity. Since such an electrode is by no means as constant as the 0.1 N calomel electrode the electrode used was connected with five similar electrodes so that drifts of potential could be detected. The second step consisted in measuring the potential of the saturated electrode against the average of six 0.1 N calomel electrodes.

The hydrogen electrode vessels were the type described previously by one of us (8). Before each measurement, the vessel was thoroughly rinsed with several portions of the solution to be tested, and a vigorous stream of hydrogen was then bubbled through the solution in the vessel. The hydrogen bearing upon the solution was then brought to atmospheric pressure, the drain-cock was closed, and the vessel shaken from 10 to 15 minutes. Shaking was then stopped and the liquid contact with the saturated potassium chloride solution was made. The potential was measured immediately with the electrode fully immersed. This first-measured potential was always taken as the significant value. Readings were continued for 5 minutes in order to make sure that any drift which might occur was small and regular. A duplicate determination was then made with a second vessel and second electrode. If, as seldom happened, the two determinations were more than 0.05 millivolt apart, measurements were repeated until a series of consistent values were obtained.

Diffusion Potentials.—The diffusion potential between saturated and 0.1 N KCl solutions was neglected. Therefore, the potential of the electrode described as Hg, HgCl, KCl (0.1 N) is really the half cell Hg, HgCl, KCl (0.1 N) / Saturated KCl.

The diffusion potential at the liquid contact with the solution in the hydrogen electrode vessel was reduced by making this contact with saturated potassium chloride solution. The Bjerrum (4, 5) extrapolation was found by essentially the same method employed by Walpole (38) in his studies of the acetic acid-sodium acetate mixtures.

Since Bjerrum himself showed that this extrapolation does not furnish values in perfect accord with those deduced from theory, and developed a theoretical explanation of why they could not,

and since Bjerrum and others have shown that the observed diffusion potentials are to some extent a function of the manner of the formation of the liquid juncture, we regard our extrapolations merely as indicating the order of magnitude of the correction to be applied.

Calomel Electrodes.—The mercury used in the calomel electrodes was thrice distilled by Hulett's (20) method and filtered between distillations. It was then sprayed repeatedly through a fine mesh cloth into a column of dilute nitric acid, washed with water in the same manner, and dried. It was then distilled twice more from a Hulett still and finally distilled *in vacuo*. The original mercury was part of a stock which had been repeatedly distilled for use in vacuum pumps.

The calomel was prepared as follows: Mercurous nitrate was formed by the action of redistilled nitric acid upon a large excess of the purified mercury. The product was thrown with the excess mercury into about 5 liters of "conductivity water" and calomel precipitated by the addition of dilute redistilled hydrochloric acid. Calomel cells prepared previously from calomel which had been washed with suction had not agreed as well as was expected, so the calomel, in this instance, was washed entirely by decantation in order to avoid change in grain size. Since washing by decantation is a very inefficient process it was continued for several days—in the preparation of 0.1 N calomel electrodes for several weeks—with renewals of 0.1 N KCl solution.

The KCl solution used for the calomel electrodes was prepared from Kahlbaum's *zur Analyse KCl*. The salt was recrystallized three times from "conductivity water" of specific conductivity of about 1×10^{-6} reciprocal ohms, ignited, and weighed with standardized weights with correction for air displacement. The solution was made up with "conductivity water" saturated with calomel containing a little mercury, and preserved in the dark in Jena bottles.

The 0.1 N calomel electrode vessels were made entirely of glass with no rubber stoppers.

Weston Cells.—Two Weston standard cells loaned by the Bureau of Standards were used. We are indebted to Mr. Shoemaker for the standardization of these cells. They were frequently used to check the commercial Weston cell which served as the working standard.

Electrodes.—Experience and the comments of others have taught us the advantage of freshly blacked electrodes when working with protein solutions. We have, therefore, adopted the use of palladium black instead of platinum black because the palladium can be dissolved off by electrolysis and there is no need to spoil the surface of the electrode with the heroic blast lamp treatment sometimes used. This was the sole reason for using palladium instead of platinum black in this investigation. The palladium black was deposited in some instances upon gold, in others upon platinum. We have found no difficulty in preparing electrodes which when thoroughly saturated with hydrogen and placed in the same solution agree within 0.02 millivolt or less. Their agreement in one solution, however, has seemed to us to be no certain guarantee of their agreement in another. Therefore, they were not compared apart from the main measurements but were assumed to agree when measurements of the same solution made with two electrodes in different vessels agreed.

We have made no search for the best means of depositing palladium black but have had good success by electrolysis, with a four volt storage battery, of a 3 per cent solution of palladium chloride containing a mere trace of lead acetate. The deposition should be carried far enough to produce a distinctly black coat but not so far that a thick layer is formed. No electrode was ever used unless the hydrogen liberated during the electrolysis in dilute sulfuric acid came off in perfectly even, small, and uniformly distributed bubbles. It was required that the deposit should be adherent enough to withstand a thorough washing with a vigorous jet of water.

Hydrogen Electrode Vessels.—These were of the type described in a previous paper (8). It may be reiterated here that the two chief principles which have been applied are: (1) the thorough establishment of the equilibria between hydrogen, electrode, and solution by shaking; and (2) the establishment of these equilibria within the vessel before the liquid contact is made so that the initial potential of the chain, before drifts of liquid diffusion potential set in, is significant. The first principle is Hasselbalch's (17); the second was suggested by the observations of Lewis and Rupert (22), Cumming and Gilchrist (12), and others on drifts of diffusion potentials. It should also be emphasized that the

electrode was fully immersed during a measurement but entirely exposed alternately to hydrogen and solution during the shaking.

Hydrogen.—Hydrogen was generated electrolytically from a 10 per cent NaOH solution, and was liberated from a nickel electrode. It was passed over sulfuric acid to take up the spray and then over heated platinized asbestos to burn out residual traces of oxygen. The generator was always kept in operation, on a low current over night, to avoid back diffusion of oxygen.

Barometer Correction.—Barometer readings were made with an instrument corrected by the Bureau of Standards. The readings were corrected for temperature, instrumental error, scale, and latitude. The pressure of hydrogen was considered to be that of the corrected barometer less the vapor tension of the solution as of pure water. The difference of pressure between that at the vessel and that at the hydrogen escapement was neglected since this escapement was through a free passage constricted for only 6 inches to about 3 mm. At times the influence of this constriction was made evident by back pressure in the hydrogen generator but it seldom if ever produced an excess pressure of over 0.2 mm. Hg.

Hydrogen electrode potentials were corrected to the *standard concentration* of hydrogen, which, contrary to the generally accepted standard in hydrogen electrode work, we have considered to be the concentration which the gas would have at 0°C. and 760 mm. corrected pressure.

Temperature Control.—An air bath was used for the maintenance of constant temperature. It consists of a well made cork-insulated box with an interior box placed so that there is an air chamber between it and the outer box on all sides but the front. A Sirocco centrifugal fan placed at one end draws air through this interior box and sends it back through the air chambers to the other end. In this way the air stream is kept free from serious eddy currents. The fan is belt-driven from the exterior.

Manipulations are made through two hand-holes with sliding doors. Light is furnished through a double window. The radiant energy of the electric bulb is cut down by a screen of nickel sulfate solution.

The heating coil is of No. 30 bare nichrome wire strung between asbestos board supports about the fan where it receives the most

vigorous air current. The electric current passing through this coil is adjusted in accordance with the room temperature so that the rate of heating when the current is thrown on by the regulator is approximately equal to the rate at which the air cools when the heat is off.

The thermoregulator is of the very sensitive type described in a former note (7)

The temperature maintained during these experiments was 20°C. according to the indications of a well seasoned thermometer calibrated by the Bureau of Standards and certified to be accurate to ± 0.02°C. Richards' point was taken by means of carefully recrystallized sodium sulfate and the thermometer found to be correct at that point within the above limit. Smaller fluctuations were estimated by means of a tapped Beckmann thermometer. Previous experiments with a thermo element had shown the temperature to vary ± 0.003° as the heat went on or off. This variation was about a very slowly drifting mean. Because of the great variation in the temperature of the room, 11–18°C., the drifts were somewhat larger during the period of these experiments than in previous work at 30°, but they seldom exceeded ±0.05° at any time; in the great majority of cases they were very much less, and during measurements were not over ±0.03°.

All solutions were kept in the bath at least 2 hours before measurement. They were held in Jena glass Erlenmeyer flasks covered tightly with tinfoil.

Potentiometer Equipment.—Potential measurements were made with a Leeds and Northrup potentiometer calibrated by the Bureau of Standards. The rubber plate on which the coils and switches are mounted has been kept clean and free from the effect of light. The slide wire is frequently cleaned by gentle rubbing with a soft vaselined cloth. The galvanometer is a Leeds and Northrup type HN instrument. It is mounted against a solid brick wall with rubber supports. The telescope support was removed from the galvanometer case and remounted out of contact with the galvanometer. With these precautions all serious tremors were avoided. All wire connections on the switch board are soldered and the copper switches kept dusted.

Wires are heavily insulated and the system is shielded. No significant thermoelectromotive forces or leaks have been detected.

The Solutions.

In the preparation of all solutions volumetric flasks with volume at 20°C. certified by the Bureau of Standards were used. These flasks were, of course, treated with care and were never heated or undercooled. Standardized weights were used in weighing solid substances, but, with the exception of the KCl for the calomel electrodes, corrections for displacement of air were not made.

The water used in the preparation of all solutions had a specific conductivity of about 1×10^{-6} reciprocal ohms.

Acid Potassium Phthalate.—This salt was made by the method of Dodge (14) from sublimed phthalic anhydride and C.P. KOH. It was recrystallized twice from distilled water and thrice more from "conductivity water" and dried at 110–115° in platinum for 2 days. 0.2 M solutions were made by dissolving 40.828 gm. in "conductivity water" and making the solution up to 1 liter at 20°C.

Acid Potassium Phosphate.—A C.P. grade of this salt was recrystallized several times from distilled and from "conductivity water." It was dried 2 days in platinum at 110–115°C. 0.2 M solutions were made by dissolving 27.231 gm. in "conductivity water" and making up to a liter at 20°C.

Boric Acid.—This was recrystallized several times, dried between filter paper during 2 dry winter days, and the constancy of weight determined with small samples held for 2 weeks in a desiccator over $CaCl_2$. 0.2 M solutions were prepared by dissolving 12.4048 gm. in "conductivity water" and making up to 1 liter at 20°.

Potassium Chloride.—Kahlbaum's *zur Analyse KCl* was recrystallized twice from "conductivity water" and ignited in platinum. The saturated solution used to reduce contact potentials was not made from the recrystallized salt but from Kahlbaum's C.P.

Boric Acid-Potassium Chloride Solution.—This solution contained 12.4048 gm. H_3BO_3 and 14.912 gm. KCl in 1 liter.

Sodium Hydroxide.—100 gm. of a high grade "NaOH from Na" were dissolved in 100 cc. of water. The Jena glass vessel was stoppered and allowed to stand about 18 hours until the precipitated carbonate had mostly settled. Instead of letting it stand longer in the glass and pipetting off the clear hydroxide solution as

Sörensen (36) recommends, it was filtered with suction through a paper[2] which had been treated as follows: A hardened filter paper was placed for half an hour in warm 1 : 1 sodium hydroxide solution. The alkali solution was decanted and the paper washed first with absolute alcohol and then with more dilute alcohol, and finally with suction by copious quantities of distilled water. The paper, now on a small Buchner funnel under gentle suction, was allowed to dry partially but not to such an extent that it began to curl. The concentrated sodium hydroxide solution was then poured upon the middle, spread with a glass rod, and gently sucked through. The resulting filtered solution appeared perfectly free from suspended carbonate. No special precautions were used to prevent access of CO_2 while diluting this solution except that exposure was made as short in duration as possible. It was diluted first to about a 2 N solution in a stoppered bottle. This was standardized roughly and the solution was then diluted to approximately 0.2 N. This was then poured quickly into a *paraffined* bottle and guarded with soda lime tubes. The burette was permanently connected with this bottle but without rubber connections.

The resulting 0.2 N solution contained so little carbonate that 50 cc. when run into a boiled barium chloride[3] solution in a flask protected by a Bunsen valve gave only a very slight cloudiness which could sometimes be seen only by using a ray of sunlight. If the flask was then opened to the laboratory air, a marked cloudiness developed in a few minutes.

This method of preparing "carbonate-free" sodium hydroxide solution is simple enough to be used in any laboratory, and it furnishes solutions which are doubtless pure enough for the present purpose.

[2] Mr. T. C. Trescot of the Bureau of Chemistry has for several years been using paper to filter concentrated sodium hydroxide solutions required in the determination of nitrogen by the Kjeldahl method. Dilute alkali solutions will, of course, disintegrate untreated paper; concentrated solutions will doubtless dissolve a small proportion of the paper treated as described, but there is no reason to believe that the organic matter carried into the solution during the time of filtration will be appreciable.

[3] This test is not of extreme delicacy but serves to show that sodium hydroxide solutions prepared as described are relatively free from carbonate.

Hydrochloric Acid.—A redistilled 20 per cent solution was diluted approximately to 0.2 N with "conductivity water."

Standardization of Solutions.—The phthalate, phosphate, and boric acid-potassium chloride solutions were standardized by weight.

The sodium hydroxide solution was standardized by titrating weighed quantities of acid potassium phthalate in a current of CO_2 free air, using phenolphthalein as the indicator.

The hydrochloric acid was standardized by weighing as silver chloride.

The hydrochloric acid and sodium hydroxide solutions were then compared by titrating the acid with the alkali in a current of CO_2 free air using phenolsulfonephthalein as the indicator.

In this way the normality of the sodium hydroxide solution was found to be:

By phthalate	0.18597
By HCl (AgCl)	0.18589

The proper factors for the hydrochloric acid and sodium hydroxide solutions were used so that results could be stated in terms of exact 0.2 N solution.

Preparation of Mixtures.—The acid potassium phthalate, acid potassium phosphate, and boric acid-KCl solutions were measured from clean calibrated pipettes into a 200 cc. flask. The 0.2 N HCl or 0.2 N NaOH, as the case might be, was then delivered from a calibrated burette, the mixture diluted to 200 cc. with "conductivity water," poured into a Jena Erlenmeyer flask, capped tightly with tinfoil, and placed in the air bath at least 2 hours before measurement. Since the ratio of acid to base is important, care was taken that one solution was not appreciably warmer than the other when measured; but since very slight dilutions of the mixture have no appreciable effect upon the hydrogen ion concentration, no particular care was taken to bring the solution exactly to 20°C. on dilution.

Accuracy of Measurements and Values Involved.

The potentials are given to the fifth decimal place because in most cases the observed potentials agreed within about 0.02–0.03 millivolt of the mean. In the subsequent calculation of the

hydrogen electrode potentials and P_H values, there are involved the theoretical potential of the 0.1 N calomel electrode referred to the normal hydrogen electrode as the standard or null point, the deviation therefrom of the particular calomel electrodes used in this investigation, and the correction to be applied for diffusion potentials. Let us first consider diffusion potentials.

Uncertainties of Diffusion Potentials.—In the older work and much of the later work, the corrections for diffusion potentials have been deduced from theoretical considerations embodied in equations such as those of Planck (34), Henderson (18), Cumming (10, 11), and Lewis and Sargent (21). Bjerrum (4, 5), however, developed the use of an interposed saturated solution of potassium chloride, and showed, not only that the diffusion potential is reduced, but that a correction may be applied by an extrapolation estimated from the potentials observed when the interposed solution is in one case saturated and in the next case half-saturated potassium chloride solution.

It should be remembered, however, that Bjerrum's method does not correct exactly, and Bjerrum himself called attention also to variations in diffusion potential, variations whose observation by Chanoz (6) apparently led Henderson (18) to revise the formula for diffusion potential. These variations or drifts have been reported by Weyl (39), Bjerrum (4), Lewis and Rupert (22), Cumming and Gilchrist (12), Walpole (38), and others.

Bjerrum concluded that the time change should be inversely proportional to the thickness of the mixed layer between adjacent solutions. Cumming and Gilchrist (12) found their own experiments to be "in general accord with Bjerrum's even though there are still some points of difficulty." They concluded, however, that the values deduced by means of one or another formula are not realized experimentally.

Our own somewhat limited observations on diffusion potential have brought us into entire agreement with the suggestions of Cumming and Gilchrist, which are as follows:

> "1. To obtain accurate measurements of potential it is necessary to form a new boundary shortly before the measurement is taken; otherwise a serious error may be introduced.
>
> 2. It is desirable that the two solutions should be mechanically mixed at the boundary and this is the more important the narrower the tube in which the boundary is formed.

3. Capillary tubes should be altogether avoided in the construction of an electromotive cell.

4. The electromotive force of any cell which derives part of its potential from the presence of a liquid contact is not constant but varies with the time which has elapsed since formation of the boundary; if due attention is paid to the precautions mentioned above, constant and comparable results may be obtained."

Myers and Acree (32) seem to have arrived at a different point of view. They were doubtless aware of the fact that the drift in diffusion potential had been observed by others with systems in which the only variation which occurred was shown to be the potential at the liquid contact. Myers and Acree, however, state:

"Several investigators, and especially Bjerrum, have observed that in measuring any system in which the hydrogen electrode is a part there is a change in the value obtained at the beginning and an hour later, perhaps. . . . . If the systems 0.1 N KCl—HgCl—Hg and 0.1 N HCl—Pt—H_2 are to be compared, it is possible to connect them by satisfactory means and still obtain a changing value for the system if it is not first saturated with hydrogen. But suppose that the connecting system[4] is first saturated with hydrogen, or, in other words, equilibrium is established and the systems are connected. Observations over periods of even a couple of hours show no changes whatever, provided no barometric change has taken place."

It may be significant to note that Cumming and Gilchrist have given a theoretical reason for believing that the time change should be small for the particular ratio of KCl to HCl mentioned in the above quotation, and that the time change would be small if the liquid contact were made in a large tube.

With the above quotation in mind, but with an entirely different purpose in view, the hydrogen electrode vessel described in a previous paper (8) was designed so that the equilibria within the vessel could be established before the liquid contact was made. In spite of this, we have found it impossible to obtain constant and at the same time reproducible potentials with hydrochloric acid solutions when a saturated solution of potassium chloride was used as the connecting fluid. A fair degree of repro-

[4] Dr. Myers tells us that by saturation of the "connecting system" with hydrogen the interposed potassium chloride solution was not meant, but only the solution whose hydrogen ion concentration was to be measured. A portion of this becomes part of the "connecting system."

ducibility could be obtained with hydrochloric acid solutions if the potential was measured directly after the formation of the liquid contact. This has been found to hold true for all the solutions tested. After this first reading a drift of potential has always been observed. The magnitude of this drift is comparatively insignificant in most cases, but with hydrochloric acid it is distinct and serious. In general the rate of drift is proportional to the magnitude of the diffusion potential estimated by the Bjerrum extrapolation.

It might be urged that this large drift with hydrochloric acid solutions was due to contamination of the solution at the electrode by the diffusion into the vessel of potassium chloride. This will not explain why potentials showed less change with more dilute hydrochloric acid solutions (the difference in density between 0.01 N HCl and 0.002 N HCl could play little part in 5 minutes), or why only negligible changes were observed with other solutions, or the very good parallelism between the magnitude of this drift and the drift observed when the contact was formed between constant calomel electrodes.

Experimental work like that of Cumming and Gilchrist is invaluable, for the biochemist must perforce study solutions whose diffusion potentials cannot be calculated. He must therefore depend upon the experimental reduction of diffusion potentials by some means such as the use of an interposed saturated solution of potassium chloride, and he can gain little help in the numerous papers on the calculation of diffusion potentials unless these lead, as did Bjerrum's work, to a basis for further correction. The Bjerrum extrapolation, however, was worked out with hydrochloric acid solutions and we know of no good reason why it should be considered applicable to all sorts of solutions except as a probable index of the magnitude of the uneliminated diffusion potential.

Nevertheless, if the magnitude of the Bjerrum extrapolation and the rate of drift in diffusion potentials are good criterions, many of the solutions used by the biochemist, whether buffer mixtures or biological fluids, have much smaller diffusion potentials against a saturated solution of potassium chloride than hydrochloric acid solutions.

The considerations stated above point to the conclusion that hydrochloric acid solutions are not well suited to routine hydrogen electrode standardization of calomel electrodes—a point to which we shall return later. Such solutions, however, have been used almost exclusively in determining the theoretical potential of the 0.1 N or normal calomel electrode in terms of the normal hydrogen electrode. Let us consider briefly some of the values given in the literature, touching first upon the definition of the "normal hydrogen electrode."

The Normal Hydrogen Electrode.—The normal hydrogen electrode is commonly supposed to be a platinized electrode immersed in a solution normal with respect to hydrogen ions and under an atmosphere of pure hydrogen and water vapor with the hydrogen at a partial pressure of 760 mm. One of us (9) will suggest in a paper now in manuscript that the definition should be revised to specify that the hydrogen gas should be at the *standard concentration* which is the concentration which the gas would have if it were under 760 mm. pressure and at 0°C.

This additional correction for the temperature of the hydrogen, like the correction for displacement of hydrogen by the vapor of a solution, is of no importance in standardizing the calomel electrode by means of a hydrogen electrode, provided the values used in any investigation all exclude or all include the correction and are all derived from data established at the temperature under consideration. But in comparing data derived at different temperatures it should be noted that the correction for both the displacement of hydrogen by water vapor and the rarefaction of the hydrogen due to its temperature is more than 0.8 millivolt greater at 30° than at 18°, and that at 30° and 760 mm. the total correction for vapor tension and temperature amounts to 1.92 millivolts.

With this in mind let us then compare some values assigned to the 0.1 N calomel electrode at different temperatures.

Values Assigned to the 0.1 N Calomel Electrode.—In Table I will be found some values referred to the normal hydrogen electrode under 760 mm. pressure of "wet" hydrogen. These have been corrected first to the normal hydrogen electrode under a partial pressure of 760 mm. hydrogen and then to the normal density or concentration of hydrogen at 0°C. and 760 mm. pressure.

TABLE I.

Potentials of the 0.1 N KCl calomel electrode referred to the normal hydrogen electrode at different temperatures and different standards of hydrogen concentration.

Author.	Temperature.	Potential referred to normal hydrogen electrode at indicated concentration of hydrogen. 760 mm.		
		"Wet" at $t°$.	"Dry" at $t°$.	"Dry" at 0°.
	°C.	volts	volts	volts
Bjerrum	0	0.3366	0.3367	0.3367
Sörensen and Koefoed	18	0.3377	0.3380	0.3388
" " "	20	0.3375	0.3378	0.3387
Clark	20	0.3373	0.3376	0.3385
Bjerrum	25	0.3367	0.3371	0.3382
Sörensen and Koefoed	30	0.3364	0.3370	0.3383
" " "	40	0.3349	0.3359	0.3378
" " "	50	0.3326	0.3344	0.3368
" " "	60	0.3290	0.3321	0.3350
Bjerrum	75	0.3243	0.3315	0.3351

It is worthy of notice that the difference of 1.3 millivolts between Sörensen and Koefoed's (35) values at 18 and 30° reduces to 0.5 millivolt when fully corrected, and that the difference of 12.3 millivolts between Bjerrum's measurements 75° apart reduces to 1.6 millivolts when the full correction is applied.

All our measurements have been corrected to 760 mm. partial pressure of hydrogen at 0°. In a sense, it may seem superfluous to make all these corrections for biochemical purposes, but the correction for changing barometer is necessary, and, when once the barometric reading has received the proper corrections, all the others can be found from a plotted curve in one operation.

For the convenience of others, we have tabulated in Table II the full corrections to be applied at different temperatures and barometric readings. These can be plotted so that the application of the correction is easy.

We have included in Table I only the measurements of Bjerrum and of Sörensen and Koefoed because they appear to be comparable enough to use in the foregoing discussion.

Loomis and Acree (27) have presented a choice of values at 25° for the 0.1 N calomel electrode which depend upon the per-

TABLE II.

Barometric corrections for hydrogen electrode potentials, including correction for vapor tension of solution (as of pure water) and correction for temperature of hydrogen.

Observed barometric pressure corrected to mm. Hg. at 0° C. and 45° latitude.	Correction. Temperature.		
	20° C.	25° C.	30° C.
mm.	*mv.*	*mv.*	*mv.*
780	0.85	1.19	1.57
770	1.02	1.36	1.74
760	1.19	1.53	1.92
750	1.36	1.71	2.10
740	1.53	1.89	2.28

centage dissociation attributed to hydrochloric acid solutions and upon the diffusion potential accepted. Loomis (26) is inclined to accept the value 0.336. The careful comparison of many electrodes by Acree and his students (13, 27, 32) together with the fact that Myers and Acree were confident enough of their hydrogen electrode potentials to express them to the sixth decimal place[5] would incline one to accept their values of the calomel cell. On the other hand, they have so far not published the details of their diffusion potential experiments, and although they used saturated KCl solution in some instances to reduce diffusion potentials they have not applied the Bjerrum extrapolation.

Lewis (23) has set forth some good reasons for believing that the hydrogen ion activity of hydrochloric acid in aqueous solutions is considerably smaller than the values generally used, and he has called attention to the fact that disagreement concerning these values will lead to serious differences in the potential of any electrode when stated in terms of the normal hydrogen electrode. Partly because of Lewis' revision of the percentage dissociation of hydrochloric acid solutions and partly because of the values given to diffusion potentials, Lewis and Randall (24) have been led to assign the value 0.2776 to the normal calomel electrode in place of 0.284 given by the "Potential Commission." Applying

[5] The barometer corrections applied in the work of Loomis and Acree, and Myers and Acree are twice the true values, as has been pointed out by Loomis (26).

directly their value 0.0530 for the difference in potential between the normal and 0.1 N calomel electrodes at 25°, we obtain 0.3306 for the value of the 0.1 N electrode in place of the commonly accepted value 0.337. The value 0.337 or values very close to it have been used in an enormous number of biochemical investigations. Fortunately, almost every one has adopted the Sörensen (36) method of expressing the results of hydrogen electrode determinations and has left the data in terms of P_H. This is simply $-\log C_H$. This value is a linear function of the potential of the hydrogen electrode and may therefore be easily corrected when new fundamental values are firmly established. There are very few instances, however, where such a revision is entirely necessary. In the great majority of cases, the biological significance of hydrogen ion concentration lies only in relative differences and the exact value assigned to the calomel electrode is unimportant, provided the same value is accepted by all and with the realization that it is somewhat arbitrary.

The older work on the calomel electrode was reviewed in 1912 (1). The provisional values assigned to the normal and 0.1 N calomel electrodes in this report were 0.284 and 0.337 respectively. Auerbach (2), reviewing the subject, gives the greatest weight to Bjerrum's average 0.3367 at 25°. He points out that this value is referred to the normal hydrogen electrode under 760 mm. pressure of "wet" hydrogen. When the correction for vapor tension is applied so that the value may be referred to the normal hydrogen electrode under 760 mm. partial pressure of hydrogen, Auerbach obtains the rounded value 0.337.

If the fully corrected values given in Table I for 18–30°, the range of ordinary experimentation, are reduced in each instance to the standard of 760 mm. partial pressure of hydrogen at $t°$ so that they will be upon the same basis used by the Potential Commission, we find that their average is 0.3375. This is only 0.4 millivolt above the value 0.3371, which Auerbach rounded off to 0.337.

Our own values determined by measurements against 0.01 N and 0.05 N HCl were vitiated, as we have already stated, by uncertainty in the liquid diffusion potential. The average of several determinations with each of six different 0.01 N HCl solutions and four measurements with two 0.05 N is 0.3385 with

the full correction for the hydrogen pressure. This happens to be identical with the average of Bjerrum's and Sörensen's corrected values at 18–30° (Table I), but the maximum variation from the mean was 0.22 millivolt and the mean variation 0.14, and the diffusion potential had to be averaged from a large number of measurements, some of which differed by 0.4 millivolt. The details of these measurements are hardly worth publishing since no very illuminative order was found in the conduct of the diffusion potentials.

The value 0.3385, referred to a normal hydrogen electrode under standard concentration of hydrogen gas (760 mm. at 0°C.), is the value we have assigned to the average of our 0.1 N calomel electrodes. It is a somewhat arbitrary value based on the considerations presented above and upon the older values for the dissociation of hydrochloric acid solutions. If it differs radically from Lewis and Randall's value and from the values given by Loomis and Acree, it is, at least, a value in harmony with those generally accepted in biochemical work, and places our data on the "buffer mixtures" in harmony with a scale of hydrogen ion concentration whose importance has become too great to justify hasty resetting.

It should be emphasized again that it is to a large extent an arbitrary value which will have to be revised when the true value shall have been so well established that there will be no hesitancy in shifting the scale used in biochemistry.

In addition to the unsatisfactory state of the question of the theoretical value to be assigned to the 0.1 N calomel electrode, we find a vexatious uncertainty in the potentials of our particular individual calomel electrodes. In the pressure of other researches we have discarded some sixty electrodes because they drifted apart sometimes as much as half a millivolt. In this investigation one of the six electrodes persisted in a potential 0.2 to 0.3 millivolt higher than the others. These other five differed among themselves 0.02 to 0.08 millivolt. We have been persistently defeated in the effort to prepare calomel electrodes agreeing better than the six used in the present investigation, and we are inclined to believe that we can improve the agreement only by adopting the Lipscomb and Hulett (25) method of preparing the calomel electrolytically. In view of Ellis' (15) excellent results with this method applied to HCl calomel electrodes, it is hardly

worth while to state more than the above summary of the disagreement of our electrodes since this is sufficient to indicate our order of accuracy. The device used by Myers and Acree (32) which consists in short circuiting differences in potentials was not resorted to.

The Measurements.

The measurements are given in Tables III to VI.

At the head of each table will be found the composition of the mixtures. In the first column are given the cc. of acid or alkali added as indicated in the heading. Then follows the average observed potential of the chain:

Hg,HgCl / Saturated KCl / Solution, Pt,H_2.

This potential is next corrected to the standard concentration of hydrogen at 0° and 760 mm. pressure with the assumption that the vapor pressure of the solution is that of pure water at the temperature used. The potential is then corrected to the basis of the average of the six 0.1 N calomel electrodes, and finally it is corrected for the diffusion potential by Bjerrum's extrapolation. The next to the last column contains the corrected hydrogen electrode potential found by subtracting the assumed standard potential of the 0.1 N calomel electrode, and finally in the last column is given the calculated value of Sörensen's P_H or $-\log [C_H]$.

In this calculation the familiar equation was used:

$$\Pi = \frac{RT}{nF} \ln \frac{1}{[C_H]}$$

R = Gas constant in volt coulombs (8.31574).
T = Absolute temperature, 273.09 + $t°$.
n = Valence of hydrogen, 1.
F = The Faraday, 96,500 coulombs.
ln = Natural logarithm. Factor for transposing to Briggsian logarithm, 0.4343.

At 20°C. the above equation becomes:

$$\Pi = 0.058155 \log_{10} \frac{1}{[C_H]} = 0.058155\ P_H$$

TABLE III.

Hydrogen electrode measurements of *phthalate-HCl mixtures* at 20° C. Composition of solutions: 50 cc. 0.2 M $KHC_8H_4O_4$ + X cc. 0.2 N HCl. Diluted to 200 cc.

X	E. M. F. observed.	E. M. F. corrected for barometer.	E. M. F. corrected to average 0.1 N calomel electrode.	E. M. F. corrected for diffusion potential.	E. M. F. corrected − 0.3385.	P_H
	volts	*volts*	*volts*	*volts*	*volts*	
0	0.47995	0.48154	0.56976	0.56938	0.23088	3.970
1	0.47620	0.47779	0.56601	0.56564	0.22714	3.906
5	0.46202	0.46319	0.55139	0.55104	0.21254	3.655
10	0.44697	0.44814	0.53634	0.53602	0.19752	3.396
15	0.43499	0.43616	0.52436	0.52405	0.18555	3.191
20	0.42450	0.42568	0.51388	0.51358	0.17508	3.011
25	0.41496	0.41614	0.50434	0.50405	0.16555	2.847
30	0.40590	0.40708	0.49528	0.49501	0.15651	2.691
35	0.39698	0.39816	0.48636	0.48611	0.14761	2.538
40	0.38830	0.38948	0.47768	0.47745	0.13895	2.389
45	0.37992	0.38110	0.46930	0.46909	0.13059	2.246
50	0.37226	0.37344	0.46164	0.46144	0.12294	2.114

TABLE IV.

Hydrogen electrode measurements of *phthalate-NaOH mixtures* at 20° C. Composition of solutions: 50 cc. 0.2 M $KHC_8H_4O_4$ + X cc .0.2 N NaOH. Diluted to 220 cc.

X	E. M. F. observed.	E. M. F. corrected for barometer.	E. M. F. corrected to average 0.1 N calomel electrode.	E. M. F. corrected for diffusion potential.	E. M. F. corrected − 0.3385.	P_H
	volts	*volts*	*volts*	*volts*	*volts*	
0	0.48051	0.48156	0.56977	0.56939	0.23089	3.970
1	0.48421	0.48526	0.57347	0.57308	0.23458	4.034
2	0.48790	0.48897	0.57718	0.57678	0.23828	4.097
5	0.49806	0.49913	0.58734	0.58692	0.24842	4.272
10	0.51207	0.51316	0.60137	0.60093	0.26243	4.513
15	0.52330	0.52439	0.61260	0.61214	0.27364	4.705
20	0.53308	0.53435	0.62253	0.62206	0.28356	4.876
25	0.54242	0.54369	0.63187	0.63139	0.29289	5.036
30	0.55205	0.55333	0.64151	0.64103	0.30253	5.202
35	0.56260	0.56388	0.65206	0.65158	0.31308	5.383
40	0.57563	0.57691	0.66509	0.66461	0.32611	5.608
45	0.59583	0.59711	0.68529	0.68480	0.34630	5.955
47	0.61009	0.61137	0.69955	0.69905	0.36055	6.200
48	0.62099	0.62229	0.71047	0.70996	0.37146	6.387
49	0.63982	0.64112	0.72930	0.72878	0.39028	6.711
50	(0.74010)	0.74140	0.82958	0.82905	0.49055	(8.435)

TABLE V.

Hydrogen electrode measurements of *"phosphate mixtures"* at 20° C. Composition of solutions: 50 cc. 0.2 M KH_2PO_4 + X cc. 0.2 N NaOH. Diluted to 200 cc.

X	E. M. F. observed.	E. M. F. corrected for barometer.	E. M. F. corrected to average 0.1 N calomel electrode.	E. M. F. corrected for diffusion potential.	E. M. F. corrected − 0.3385.	P_H
	volts	*volts*	*volts*	*volts*	*volts*	
1	0.55346	0.55468	0.64293	0.64267	0.30417	5.230
2	0.57076	0.57200	0.66025	0.65997	0.32147	5.528
5	0.59449	0.59573	0.68398	0.68366	0.34516	5.935
10	0.61423	0.61547	0.70372	0.70337	0.36487	6.274
20	0.63770	0.63894	0.72719	0.72680	0.38830	6.677
25	0.64757	0.64871	0.73686	0.73646	0.39796	6.843
30	0.65745	0.65859	0.74674	0.74633	0.40783	7.013
40	0.68142	0.68266	0.77081	0.77039	0.43189	7.427
45	0.70187	0.70314	0.79129	0.79084	0.45234	7.778
47	0.71606	0.71733	0.80548	0.80500	0.46650	8.022

TABLE VI.

Hydrogen electrode measurements of *"borate mixtures"* at 20°C. Composition of solutions: 50 cc. 0.2 M H_3BO_3, 0.2 M KCl + X cc. 0.2 N NaOH. Diluted to 200 cc.

X	E. M. F. observed.	E. M. F. corrected for barometer.	E. M. F. corrected to average 0.1 N calomel electrode.	E. M. F. corrected for diffusion potential.	E. M. F. corrected − 0.3385.	P_H
	volts	*volts*	*volts*	*volts*	*volts*	
0	(0.57400)	0.57511	0.66343	0.66343	0.32493	(5.587)
1	0.67680	0.67791	0.76623	0.76623	0.42773	7.355
2	0.69521	0.69629	0.78459	0.78459	0.44609	7.671
3	0.70633	0.70743	0.79573	0.79573	0.45723	7.862
4	0.71447	0.71557	0.80386	0.80386	0.46536	8.002
5	0.72108	0.72218	0.81047	0.81046	0.47196	8.116
10	0.74258	0.74391	0.83232	0.83230	0.49380	8.491
15	0.75720	0.75853	0.84694	0.84691	0.50841	8.742
25	0.78023	0.78156	0.86997	0.86991	0.53141	9.138
35	0.80243	0.80376	0.89217	0.89209	0.55359	9.519
40	0.81609	0.81742	0.90583	0.90573	0.56723	9.754
45	0.83566	0.83699	0.92540	0.92529	0.58679	10.090
47	0.84763	0.84896	0.93737	0.93725	0.59875	10.296
50	0.87820	0.87953	0.96794	0.96782	0.62932	10.821

TABLE VII.

P_H values of *KCl-HCl mixtures*. Preliminary measurements at 25°C. Composition of solutions: 50 cc. 0.2 M KCl + X cc. 0.2 N HCl. Diluted to 200 cc.

X	P_H	X	P_H
2	2.733	20	1.722
5	2.332	25	1.625
7	2.180	40	1.416
10	2.027	50	1.314
15	1.847		

DISCUSSION.

The observed potentials given in the tables are the average of at least two measurements agreeing within ±0.03 millivolt of the mean. The onset of warm weather has prevented repetition of these measurements at 20° with the present equipment, but they will be repeated at higher temperatures in order to establish the temperature coefficients.

0.05 M instead of the higher concentrations have been studied for two reasons. In the first place, solutions as concentrated as 0.1 M are at the border line of what may be considered the range of dilute solutions and are therefore less adapted to the theoretical treatment which may become necessary. In the second place, we have desired for our own work a series of solutions whose salt concentration is more nearly comparable with that of ordinary bacteriological culture media than are the solutions generally used.

The addition of potassium chloride to the borate mixtures was found advisable in order to make the "salt effect" upon indicators comparable in the borate and phosphate mixtures. There is a certain inconsistency in retaining the same concentration of potassium chloride as the salt in the form of borate increases. This might be overcome by dilution of the borate mixtures with declining volumes of a potassium chloride solution, but we have considered this unwise in view of the fact that careless errors in the volume of potassium chloride might introduce a larger effect upon the hydrogen ion concentration of the solution than the "salt error" of the present method. Furthermore, the more alka-

line borate mixtures where this salt error might become appreciable will seldom need to be used in bacteriological or physiological investigations. The effect of the potassium chloride upon the P_H values of borate mixtures was shown by a rise of about 0.04 P_H. Similar effects of neutral salts have been discussed by Sörensen (36), Loomis and Acree (27), Walpole (38), and Harned (40).

Since potential measurements of "unbuffered" mixtures cannot be accurately made, the potentials observed with such mixtures are bracketed.

For bacteriological colorimetric work we have found it convenient to use these mixtures at intervals of 0.2 P_H. Interpolation to 0.1 is easy in most cases and the accuracy allowed is adequate for all general work. For the convenience of those who wish to use this system we have given in Table VIII the compositions corresponding to intervals of 0.2 P_H. We make up the full 200 cc. of solution instead of the 10 cc. of other systems because one generally wishes several portions of a solution in a day's work, and because, by preserving these solutions in properly stoppered bottles each with its own 10 cc. pipette, there is always some at hand for the occasional test. All solutions should be kept in resistant glass and the borate mixtures in paraffined bottles.

It will be found that the more acid phthalate-hydrochloric acid solutions contain so much free phthalic acid that it will crystallize out at 20°. The separation of the phthalic acid probably produces an insignificant change in the hydrogen ion con-

TABLE VIII.

Composition of mixtures giving P_H values at 20°C. at intervals of 0.2 P_H.

P_H	Phthalate—HCl mixtures.		
2.2	50 cc. 0.2 M KH Phthalate	46.70 cc. 0.2 N HCl.	Diluted to 200 cc.
2.4	50 " 0.2" " "	39.60 " 0.2" " "	" " " "
2.6	50 " 0.2" " "	32.95 " 0.2" " "	" " " "
2.8	50 " 0.2" " "	26.42 " 0.2" " "	" " " "
3.0	50 " 0.2" " "	20.32 " 0.2" " "	" " " "
3.2	50 " 0.2" " "	14.70 " 0.2" " "	" " " "
3.4	50 " 0.2" " "	9.90 " 0.2" " "	" " " "
3.6	50 " 0.2" " "	5.97 " 0.2" " "	" " " "
3.8	50 " 0.2" " "	2.63 " 0.2" " "	" " " "

TABLE VIII—*Concluded.*

P_H	Phthalate—NaOH mixtures.											
4.0	50 cc.	0.2 M	KH Phthalate		0.40 cc.	0.2 N	NaOH.		Diluted	to	200	cc.
4.2	50 "	0.2 "	"	"	3.70 "	0.2 "	"	"	"	"	"	"
4.4	50 "	0.2 "	"	"	7.50 "	0.2 "	"	"	"	"	"	"
4.6	50 "	0.2 "	"	"	12.15 "	0.2 "	"	"	"	"	"	"
4.8	50 "	0.2 "	"	"	17.70 "	0.2 "	"	"	"	"	"	"
5.0	50 "	0.2 "	"	"	23.85 "	0.2 "	"	"	"	"	"	"
5.2	50 "	0.2 "	"	"	29.95 "	0.2 "	"	"	"	"	"	"
5.4	50 "	0.2 "	"	"	35.45 "	0.2 "	"	"	"	"	"	"
5.6	50 "	0.2 "	"	"	39.85 "	0.2 "	"	"	"	"	"	"
5.8	50 "	0.2 "	"	"	43.00 "	0.2 "	"	"	"	"	"	"
6.0	50 "	0.2 "	"	"	45.45 "	0.2 "	"	"	"	"	"	"
6.2	50 "	0.2 "	"	"	47.00 "	0.2 "	"	"	"	"	"	"
P_H	KH_2PO_4—NaOH mixtures.											
5.8	50 cc.	0.2 M	KH_2PO_4		3.72 cc.	0.2 N	NaOH.		Diluted	to	200	cc.
6.0	50 "	0.2 "	"		5.70 "	0.2 "	"		"	"	"	"
6.2	50 "	0.2 "	"		8.60 "	0.2 "	"		"	"	"	"
6.4	50 "	0.2 "	"		12.60 "	0.2 "	"		"	"	"	"
6.6	50 "	0.2 "	"		17.80 "	0.2 "	"		"	"	"	"
6.8	50 "	0.2 "	"		23.65 "	0.2 "	"		"	"	"	"
7.0	50 "	0.2 "	"		29.63 "	0.2 "	"		"	"	"	"
7.2	50 "	0.2 "	"		35.00 "	0.2 "	"		"	"	"	"
7.4	50 "	0.2 "	"		39.50 "	0.2 "	"		"	"	"	"
7.6	50 "	0.2 "	"		42.80 "	0.2 "	"		"	"	"	"
7.8	50 "	0.2 "	"		45.20 "	0.2 "	"		"	"	"	"
8.0	50 "	0.2 "	"		46.80 "	0.2 "	"		"	"	"	"
P_H	Boric acid, KCl—NaOH mixtures.											
7.8	50 cc.	0.2 M	H_3BO_3,	0.2 M KCl	2.61 cc.	0.2 N	NaOH.		Diluted	to	200	cc.
8.0	50 "	0.2 "	"	" "	3.97 "	0.2 "	"		"	"	"	"
8.2	50 "	0.2 "	"	" "	5.90 "	0.2 "	"		"	"	"	"
8.4	50 "	0.2 "	"	" "	8.50 "	0.2 "	"		"	"	"	"
8.6	50 "	0.2 "	"	" "	12.00 "	0.2 "	"		"	"	"	"
8.8	50 "	0.2 "	"	" "	16.30 "	0.2 "	"		"	"	"	"
9.0	50 "	0.2 "	"	" "	21.30 "	0.2 "	"		"	"	"	"
9.2	50 "	0.2 "	"	" "	26.70 "	0.2 "	"		"	"	"	"
9.4	50 "	0.2 "	"	" "	32.00 "	0.2 "	"		"	"	"	"
9.6	50 "	0.2 "	"	" "	36.85 "	0.2 "	"		"	"	"	"
9.8	50 "	0.2 "	"	" "	40.80 "	0.2 "	"		"	"	"	"
10.0	50 "	0.2 "	"	" "	43.90 "	0.2 "	"		"	"	"	"

centration. We hope to report upon measurements at higher temperatures in the near future.

The defects of this system may be summarized as follows:

1. Inasmuch as the hydrogen ion concentration of a mixture is determined chiefly by the ratio of acid to base, the substitution of NaOH in place of the Na_2HPO_4 used by Sörensen in the phosphate mixtures and of NaOH in place of $Na_2B_4O_7$ used by Palitzsch in the borate mixtures may introduce serious errors in the more alkaline mixtures of each series, if the sodium hydroxide and acid potassium phosphate or the sodium hydroxide and boric acid (KCl) are not standardized to the correct ratio.

2. Of the more acid mixtures of the phthalate-hydrochloric acid series, those more acid than $P_H = 2.6$ are saturated with free phthalic acid which will crystallize out at low temperatures.

3. The correction for the salt error of indicators by the addition of potassium chloride to the more acid borate mixtures in order to make these comparable with the phosphate mixtures is continued in the more alkaline mixtures where the salts of boric acid doubtless produce a salt effect.

The second and third criticisms will probably have little significance for the particular use for which this set of mixtures was designed. It should be emphasized that the variation in the salt concentration of the mixtures we describe is very much less than that of some of the Sörensen mixtures; for example, the citrate mixtures.

The first mentioned defect is one which applies not only to these mixtures but to Sörensen's citrate mixtures, borate mixtures, and alkaline glycocoll mixtures. It is compensated for by the fact that the sodium hydroxide may be prepared in sufficient purity and standardized with sufficient accuracy by simple means if the procedures outlined are followed, and also by the fact that all the solid substances used are easily purified, free from water of crystallization, unaffected by atmospheric carbon dioxide, and invested with many of the properties which make possible accurate preparation of their solutions.[6]

[6] In making this statement we are not unmindful of the fact that because a substance crystallizes beautifully it is no guarantee that it can thereby be perfectly freed from impurities. We believe, however, that three crystallizations of the substances mentioned, if properly done, will furnish material of a purity adequate for the purpose. We recommend that under no circumstances unrecrystallized material should be used.

Colorimetric checks upon the consistency of any particular set of these mixtures may be made by comparing "5.8" and "6.2" phthalate with "5.8" and "6.2" phosphate, using dipropyl red (28) and dibromothymolsulfonephthalein (28) as the indicators, and by comparing "7.8" and "8.0" phosphate with "7.8" and "8.0" borate, using phenolsulfonephthalein (28) and α-naphtholphthalein as the indicators. The ratio of the hydrochloric to sodium hydroxide is susceptible to accurate determination, and one of the substances used in these mixtures, namely, acid potassium phthalate, is eminently adapted to the standardization of the sodium hydroxide solution.

The system of mixtures here described is not so complete as the admirable Sörensen mixtures, but just as Palitzsch found it best to alter the borate mixtures to make their preparation possible on long ship voyages, so we believe the system here outlined will appeal to the peculiar needs of the bacteriologist if it does not find a more general acceptance.

Acid Potassium Phthalate as a Standard.

In routine hydrogen electrode measurements some solution with well defined hydrogen ion concentration is generally chosen to standardize the particular system used as well as to check the accuracy or stability of the particular calomel electrode used. Any one of the buffer mixtures whose hydrogen ion concentration is well defined may be so employed. Thus Sörensen uses a citric acid-sodium citrate mixture and Michaelis has recommended the "standard acetate mixture" which Walpole has carefully studied. Others have used hydrochloric acid solutions. The difficulties attending the use of hydrochloric acid have been mentioned by others, especially Michaelis, and have been touched upon in this paper. The "standard citrate" and "standard acetate" solutions must be constructed by adjustment of their components, and the standard acetate solution, though excellent in some respects, loses acetic acid easily.

Acid potassium phthalate, on the other hand, possesses a somewhat unique combination of properties which makes its solution an excellent standard. Examination of the "titration curve," of phthalic acid (Fig. 2) will show at a glance that the first and

second dissociation constants are so close that the second hydrogen begins to dissociate before the first is neutralized. In other words, when the mono alkali salt is formed, the solution still retains a marked "buffer effect." A solution, therefore, of the crystalline salt, unlike solutions of practically all the salts of

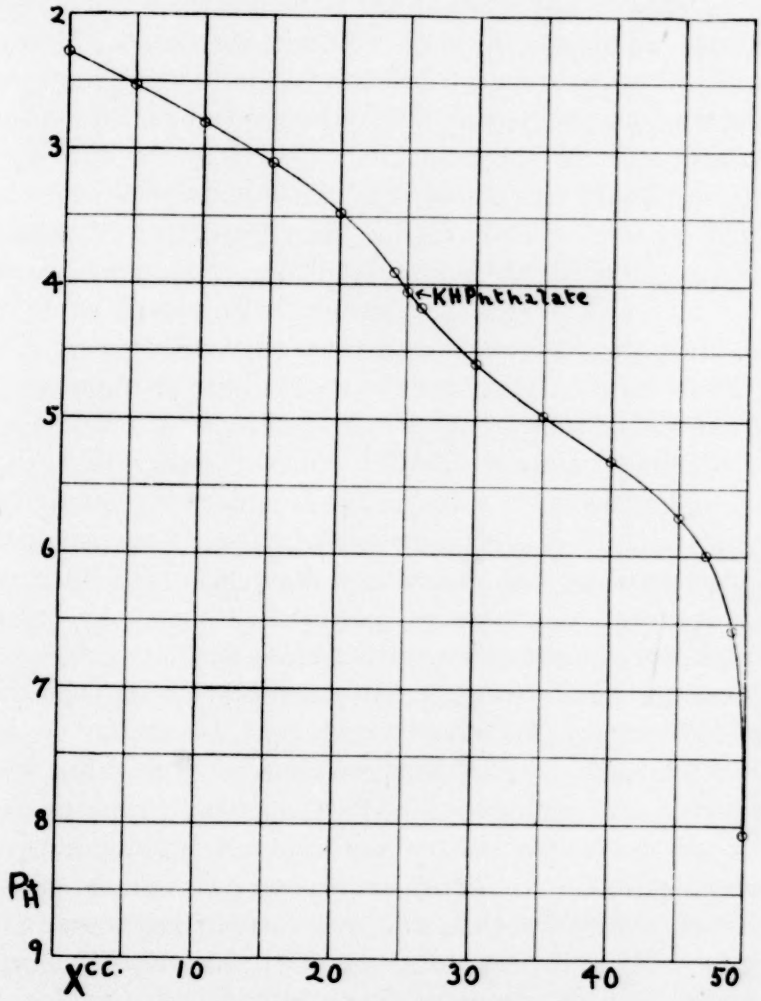

FIG. 2. Titration curve of *o*-phthalic acid at 30°. 150 cc. M/30 phthalic acid + X cc. 0.2 M NaOH. Diluted to 200 cc.

monobasic and the majority of polybasic acids, possesses a stable hydrogen ion concentration. This happens also to be sufficiently high to prevent atmospheric carbon dioxide from having much effect. When it is recalled that acid potassium phthalate can be prepared with high purity, can be easily recrystallized, and has

no water of crystallization, it would seem to be ideal for the standardization of a hydrogen electrode-calomel electrode system. Its apparent diffusion potential against saturated KCl, estimated by the Bjerrum extrapolation method, is somewhat higher than that of "standard acetate," but not seriously high. We have observed no sign of any reduction by the electrode such as occurs with sodium amalgam in very alkaline solutions. Unlike the acetic acid in "standard acetate" the phthalic acid is not volatile and therefore does not require the hydrogen to be bubbled through an elaborate train of the solution. Certainly for routine work it will be simpler to use a solution whose ratio of acid to base is determined by the crystals rather than by artful adjustment.

The comparison of phthalate solutions, "standard acetate," and other mixtures which are particularly suited to hydrogen electrode measurements can furnish us with reliable fixed points such as those used in thermometry. So long as relative values only are needed, these points would suffice to subdivide a scale which would be extremely useful. Such a scale might be made totally independent of the potential of any secondary standard such as the calomel electrode, it need have no concern with any conception regarding the percentage dissociation of hydrochloric acid, and it could, if necessary, justify itself without leaning at any point upon conductivity measurements.

On the other hand, the necessity for correlating the data of different experimental methods makes it advisable to fix the position of the scale by standardization with a solution which is experimentally susceptible to a fundamental evaluation of its hydrogen ion concentration by methods not involving hydrogen electrode potentials. In many respects hydrochloric acid solutions are well suited for this, and our scale of hydrogen ion concentrations will doubtless continue for some time to have its position fixed by measurements with hydrochloric acid.

When opportunity can be found, we hope to make a careful comparison of phthalate, hydrochloric acid, "standard acetate," and other standard solutions. Therefore, we are not presenting our value for the potential of a 0.05 M acid potassium phthalate solution at 20°, which will be found in Tables III and IV, as our final value. It is, however, probably accurate enough for all routine uses.

SUMMARY.

The hydrogen electrode potentials of the following mixtures have been studied at 20°C.

Acid potassium phthalate	—	hydrochloric acid.	
" "	—	sodium hydroxide.	
" " phosphate	—	"	"
Boric acid + KCl	—	"	"
Boric acid	—	"	"
At 25°C. potassium chloride	—	hydrochloric acid.	

In all cases the concentration of the first component was maintained at 0.05 M.

The evaluation of the 0.1 N KCl calomel electrode in terms of the "normal hydrogen electrode" is discussed.

It is suggested that a temperature correction should be included in the barometric correction of hydrogen electrode potentials. The application of this together with a consideration of several theoretical and experimental difficulties leads to the conclusion that in biochemical work the 0.1 N KCl calomel electrode should be given the provisional and somewhat arbitrary value 0.3385 between 18 and 30°C. when referred to the potential of the "normal hydrogen electrode."

By applying the full barometric correction and the above value of the 0.1 N calomel electrode the P_H values of the various mixtures were calculated, and the compositions given which furnish mixtures differing by intervals of 0.2 P_H for use as comparison solutions in the colorimetric determination of hydrogen ion concentrations.

It is shown that the system of "buffer" mixtures described is somewhat more simple to prepare and easier to standardize than the systems in common use.

Acid potassium phthalate solutions possess properties which make them comparable with or better than "standard acetate" and similar solutions for standardizing hydrogen electrode systems.

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THE CONJUGATED SULFURIC ACID OF THE MUCIN OF PIG'S STOMACH (MUCOITIN SULFURIC ACID).

FIRST PAPER.

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It has been demonstrated in recent years that a conjugated sulfuric acid combined with protein is widely distributed in animal tissues and that it is present even in leukocytes. The question of the identity or non-identity of the conjugated sulfuric acids of different origin could not be answered so long as there existed no detailed knowledge of the structure of any one substance of this group.

The work of Levene and La Forge[1] has furnished considerable information regarding the structure of the acid derived from cartilage, the chondroitin sulfuric acid discovered by Mörner[2] and first studied by Schmiedeberg.[3]

The analysis of the conjugated sulfuric acid derived from tendomucoid followed, and revealed the identity of this substance with the original chondroitin sulfuric acid. Hence chondromucoid and tendomucoid can differ only by the nature of their protein components, if they possess any point of difference at all.

Very little is known about the relationship of mucoids to true mucin. First mention of the presence of a conjugated sulfuric acid in the molecule of true mucin was made by Levene,[4] and in recent years much work on this subject was done in Hofmeister's laboratory. López-Suárez[5] has isolated a conjugated

[1] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, 1914, xviii, 237.
[2] Mörner, C. T., *Skand. Arch. Physiol.*, 1889, i, 210.
[3] Schmiedeberg, O., *Arch. exp. Path. u. Pharm.*, 1891, xxviii, 358.
[4] Levene, P. A., *Biochem. Z.*, 1909, xvi, 246.
[5] López-Suárez, J., *Biochem. Z.*, 1913, lvi, 167.

sulfuric acid from the mucin occurring in pig's stomach, and Alzona,[6] employing Levene's picric acid method, isolated the same substance from the mucous membrane of the stomach, the intestines, the prostate, the bladder, and from the thyroid.

The present work deals with the conjugated sulfuric acid obtained from the mucin occurring in the pig's stomach. The substance was prepared by the same process as described by Levene and La Forge for tendomucin.

In this phase the substance contained besides sulfuric also phosphoric acid. For further purification use was made of a process previously described by Levene; namely, the crude conjugated sulfuric acid was separated from the nucleic acids as a water-soluble barium salt. However, the nucleic acid was removed only after many repeated precipitations. The final product contained practically no phosphorus at all, but it apparently was partially hydrolyzed by the continued treatment with barium hydroxide. In its carbon: nitrogen ratio the substance came nearer to the original chondroitin sulfuric acid than the samples analyzed in Hofmeister's laboratory.

	Calculated for $C_{28}H_{41}N_2S_2O_{29}Ba_2$.	Found for substances obtained by			
		Levene.[7]	Levene and La Forge.[8]	Levene and López-Suárez.	López-Suárez[9] (in Hofmeister's laboratory).
C.....	27.80	27.29	25.13	36.08	43.29
H.....	3.48	3.64	3.88	5.32	5.47
N.....	2.32	2.58	2.11	2.89	5.37
S.....	5.30	4.85	4.26	1.85	4.29
Ba....	22.70	21.90	18.35	14.38	

The substance gave a positive test with orcin in intensity resembling that of chondroitin sulfuric acid. On distillation with hydrochloric acid the presence of furfurol was demonstrated by the aniline acetate test.

Efforts are being made to obtain the substance in a purer state. The results will be communicated in a subsequent report; the

[6] Alzona, F., *Biochem. Z.*, 1914, lxvi, 408.

[7] Levene, P. A., and Jacobs, W. A., *J. Exp. Med.*, 1908, x, 557. *Biochem. Z.*, 1909, xvi, 248.

[8] Levene and La Forge, *J. Biol. Chem.*, 1914, xviii, 239.

[9] López-Suárez, *Biochem. Z.*, 1913, lvi, 170.

present one deals with the nature of the nitrogenous component of the new conjugated acid.

The nitrogen-containing component was recognized as *d*-glucosamine. It was identified by the solubility of its hydrochloride, by the character and magnitude of its optical rotation, by the properties of its osazone, and finally by the conversion into chitonic acid.

This finding establishes the first chemical point of distinction between true mucin and mucoid. It is in harmony with the experience of F. Müller,[10] who obtained glucosamine on hydrolysis of salivary mucin.

Further work on mucoitin sulfuric acid (as we propose to name this substance) is now in progress. A survey of other mucins and mucoids is also contemplated.

EXPERIMENTAL PART.

Preparation of the Crude Conjugated Sulfuric Acid.—The mucous content of fresh pig's stomachs was dissolved in a 3 per cent solution of sodium hydroxide and allowed to stand for 48 hours. The solution was then rendered slightly acid by means of acetic acid, an excess of barium carbonate was added, and the mixture placed on the water bath, where it remained until the liquid acquired a very clear appearance. The precipitate was then removed by centrifugalization. Further treatment differed in individual experiments. In some the conjugated sulfuric acid was precipitated by means of glacial acetic acid, in others by means of lead acetate. The lead salts were converted into the free substance in the following manner. The salt was suspended in about ten parts of water, excess of barium carbonate added, and hydrogen sulfide passed through the solution. During this process the mixture was agitated by means of a mechanical stirrer, and the solution was kept at about 95°C. The filtrate from lead sulfide was concentrated under diminished pressure to about one-fourth of the original volume. To this solution an equal volume of 95 per cent alcohol was added, and in this manner the crude barium salt was obtained. The substance precipitated by means of glacial acetic acid was dissolved in water and precipi-

[10] Müller, F., *Z. Biol.*, 1901, xlii, 468.

tated by an equal volume of alcohol. The precipitate thus formed differed very little from the crude material obtained by the lead process.

Purification of the Crude Material.—The crude material contained impurities consisting principally of nucleic acid. In order to remove this the crude substance was dissolved in water, an excess of barium hydroxide solution was added, and the excess of barium hydroxide removed by a stream of carbonic acid gas. The barium salt of nucleic acid and barium carbonate were removed by centrifugalization. To the clear filtrate an equal volume of alcohol was added. The precipitate was redissolved, the solution again reprecipitated, and this was continued until the final precipitate gave an absolutely clear solution. About 80 per cent of the crude material is lost in the process of purification. For analysis the substances were dried under diminished pressure at the temperature of water vapor.

Sample I.
0.1368 gm. substance (Kjeldahl) neutralized 3.45 cc. 0.1 N HCl.
0.1524 " " gave 0.0200 gm. $BaSO_4$ (S determination).
0.1539 " " " 0.0562 " " (Ba ").
Sample II.
0.0996 gm. substance gave 0.1318 gm. CO_2 and 0.0474 gm. H_2O.
0.0952 " " (Kjeldahl) neutralized 1.97 cc. 0.1 N HCl.
0.0865 " " gave 0.0118 gm. $BaSO_4$ (S determination).
0.0908 " " " 0.0222 " " (Ba ").

	Calculated for $C_{28}H_{44}N_2S_2O_{29}Ba_2$:	Found: I.	II.
C	27.80		36.08
H	3.48		5.32
N	2.32	3.53	2.89
S	5.30	1.80	1.85
Ba	22.70	21.49	14.38

Hydrolysis of the Conjugated Sulfuric Acid.—20 gm. of the partially purified substance, taken up with 100 cc. of 20 per cent hydrochloric acid and 4.0 gm. of stannous chloride, were heated with a return condenser over flame for $7\frac{1}{2}$ hours. The solution was diluted with an equal volume of water, hydrogen sulfide passed through the solution, and the filtrate concentrated under diminished pressure (approximately 15 mm.) nearly to dryness. A crystalline sediment formed in the flask. This was transferred

to a beaker by means of methyl alcohol. The substance was recrystallized out of dilute methyl alcohol. Unlike chondrosamine hydrochloride, the substance was insoluble in methyl alcohol, and crystallized in plates resembling those of glucosamine hydrochloride. The substance did not melt. It reduced Fehling's solution, and formed a glucosazone. For analysis it was dried in a vacuum desiccator.

0.0200 gm. substance in the Van Slyke amino apparatus gave 2.38 cc. N at 25° and 757 mm.

0.1500 gm. substance by the Volhard method required 6.86 cc. 0.1 N $AgNO_3$.

	Caclulated for $C_6H_{13}O_5NHCl$:	Found:
N	6.51	6.57
Cl	16.45	16.23

The substance had the following rotation:

Initial:

$$[\alpha]_D^{20} = \frac{+4.30° \times 2.1862}{0.5 \times 0.2008 \times 1.039} = +90.1°$$

Equilibrium:

$$[\alpha]_D^{20} = \frac{+3.37° \times 2.1862}{0.5 \times 0.2008 \times 1.039} = +70.63°$$

Oxidation of the Hexosamine.—6 gm. of the sugar were dissolved in 25 cc. of water, 6.0 gm. of silver nitrite added, allowed to stand 6 hours, then another portion of 3.0 gm. of silver nitrite and the equivalent quantity of a 10 per cent hydrochloric acid solution were added. The mixture was allowed to stand over night. The solution then contained 0.0027 gm. of amino nitrogen. The excess of silver was removed by means of hydrogen sulfide. To the solution 15.0 gm. of bromine were added and allowed to stand 48 hours. The calcium salt of chitonic acid was then prepared in the usual way.

For analysis the calcium salt was dried in a vacuum desiccator at the temperature of water vapor.

0.0994 gm. substance gave 0.1212 gm. CO_2, 0.0472 gm. H_2O, and 0.0134 gm. CaO.

	Calculated for $(C_6H_9O_6)Ca+2H_2O$:	Found:
C	33.47	33.25
H	5.11	5.27
CaO	13.07	13.59

The optical rotation of the substance:

$$[\alpha]_D^{20} = \frac{+1.63^\circ \times 2.149}{1 \times 1.000 \times 1.010} = +34.67^\circ$$

Fischer[11] gives for the same substance $[\alpha]_D^{19} = +32.8^\circ$.

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[11] Fischer, E., and Tiemann, F., *Ber. chem. Ges.*, 1894, xxvii, 138.

CEPHALIN. IV.*

PHENYL- AND NAPHTHYLUREIDOCEPHALIN.

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In a previous communication we pointed out the discrepancy existing between the empirical analytical data of cephalin and the figures required by the theory, based on the assumption that the molecule contains only those components which have been identified on hydrolysis of the substance.

	C	H	N	P
Required	66.17	10.57	1.88	4.17
Found	60.00	9.38	1.68	4.27

At the time of that publication we were hopeful of obtaining convincing evidence as to the composition of cephalin by the conversion of the phosphatide into its tetrahydro derivative. However, little progress was made by that method. Various catalysts were prepared and found efficient for the reduction of unsaturated aliphatic compounds. Their efficiency was invariably disappointing when applied to the reduction of cephalin. Either the phosphatide itself or some impurity adhering to it acted as a catalytic poison, and after a brief interval of absorption of hydrogen the reaction stopped. Hence we were forced for the time to abandon the hope of preparing a satisfactory supply of the tetrahydro derivative.

At the same time it was attempted to approach the solution of the original problem from another angle. It has been conclusively demonstrated that cephalin contains a free amino group. *A priori* there is nothing to prevent this group from entering into the reactions characteristic of it. Hence cephalin, in a way,

* Levene, P. A., and West, C. J., *J. Biol. Chem.*, 1913–14, xvi, 419; 1916, xxiv, 41, 111.

may be regarded as a complex amino-acid. For these their ureido, and also their acyl derivatives were found very serviceable for the purpose of isolation and identification. It seemed logical to expect cephalin to form acyl and ureido derivatives.

This expectation was only partially realized. The attempt to prepare acyl derivatives of constant composition failed.[1] On the other hand, the phenyl- and the naphthylureido derivatives, though not crystalline, possessed satisfactory physical properties. They formed non-hygroscopic, very slightly colored powders. The phenyl derivative was reduced by means of hydrogen according to Paal's method. Each of the three substances on analysis gave results harmonizing with those calculated on the basis of the generally found composition of cephalin: $C_{41}H_{78}NPO_{13}$.

EXPERIMENTAL.

Phenylureidocephalin.

10 gm. of brain cephalin were dissolved in about 50 cc. of dry chloroform, 5 gm. of phenyl isocyanate added, and the mixture was allowed to stand at room temperature for 2 days. The diphenylurea was then filtered off and washed with a little chloroform. The filtrate and washings were concentrated on the water bath and poured, with stirring, into a large volume of dry acetone. The granular precipitate was filtered and washed with dry acetone. The ureido derivative was purified by dissolving in dry ethyl acetate and cooling the solution to 0°. It settles on as a granular amorphous powder, which is readily filtered and dried. Cephalin, under the same conditions, settles out as a sticky mass, which must be thoroughly ground up with dry acetone before it can be handled.

0.1012 gm. substance gave 0.2272 gm. CO_2 and 0.0788 gm. H_2O.
0.5000 " " (Kjeldahl) neutralized 11.35 cc. 0.1 N HCl.
0.3000 " " gave 0.0356 gm. $Mg_2P_2O_7$.

	Calculated for $C_{41}H_{78}NPO_{13} + C_6H_5CNO$:	Found:
C	61.11	61.20
H	8.87	8.70
N	2.97	3.18
P	3.29	3.30

[1] The acetyl, benzoyl, and *p*-nitrobenzoyl derivatives were studied.

Phenylureidotetrahydrocephalin.

The phenylureido compound, purified as above, was dissolved in a mixture of ethyl alcohol and acetic acid and reduced with hydrogen gas according to Paal. The reduction mixture was freed from palladium by warming and filtering, and the reduced phenylureido compound precipitated from the filtrate by dry acetone. This was purified by dissolving in dry ethyl acetate and allowing the product to settle out at 0°. It is a granular amorphous product, resembling in appearance the original urethane, but being considerably more insoluble.

0.1200 gm. substance gave 0.2702 gm. CO_2 and 0.0997 gm. H_2O.
0.2000 " " (Kjeldahl) neutralized 3.90 cc. 0.1 N HCl.
0.3080 " " gave 0.0370 gm. $Mg_2P_2O_7$.

	Calculated for $C_{41}H_{82}NPO_{13} + C_6H_5CNO$:	Found:
C	60.85	61.40
H	9.20	9.30
N	2.95	2.73
P	3.25	3.43

Naphthylureidocephalin.

10 gm. of cephalin in chloroform solution and 5 gm. of naphthyl isocyanate were allowed to stand at room temperature for 2 days and the product was isolated as described under phenylureidocephalin. The naphthylureido derivative was purified by reprecipitation from a chloroform solution by dry acetone. It differed from cephalin by its insolubility in ethyl acetate, acetic acid, and mixtures of ethyl alcohol and acetic acid, and by its slight solubility in ether. Because of this insolubility it could not be reduced.

0.1012 gm. substance gave 0.2358 gm. CO_2 and 0.0732 gm. H_2O.
0.5000 " " (Kjeldahl) neutralized 11.35 cc. 0.1 N HCl.
0.3000 " " gave 0.0338 gm. $Mg_2P_2O_7$.

	Calculated for $C_{41}H_{78}NPO_{13} + C_{10}H_7CNO$:	Found:
C	62.87	63.50
H	8.62	8.09
N	2.82	3.18
P	3.12	3.14

THE UTILIZATION OF SUCROSE AND THE INVERTING POWER OF THE BLOOD SERUM AFTER PARENTERAL ADMINISTRATION OF SUCROSE.

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(Received for publication, May 20, 1916.)

It has been assumed that sucrose, which can be easily inverted in the alimentary tract, is eliminated quantitatively in the urine, when it is introduced into the animal body parenterally. According to the recent reports of Mendel and Mitchell (1905), Heilner, Mendel and Kleiner, and Hogan, the amount of sucrose recovered in the urine is less than that injected. Mendel and Kleiner concluded that, when sucrose was introduced parenterally into dogs and cats in doses of 1 or 2 gm. per kilo of body weight, the quantity excreted usually amounted to more than 65 per cent of that introduced. The results of other investigations are not far from this. Mendel and Kleiner, and Hogan were unable to cause any better utilization of sucrose by repeated injections. The fate of the part which cannot be recovered has not been determined. Jappelli suggested that part of the sucrose might be eliminated into the alimentary tract, and then be utilized, as is customary after oral administration. Reducing sugar is sometimes found in the urine, if the amount of sucrose injected is not too small, indicating that some of the sucrose injected is inverted somewhere in the animal body.

It is generally believed that in the animal body invertin can be found only in the mucous membrane of the intestine. Weinland claimed that repeated subcutaneous injections of sucrose cause an invertin to appear in the serum of puppies. He found, moreover, that the power to utilize sucrose increased with repeated injections. In his experiments, quantitative measurements were not attempted. Weinland explained his results by assuming that the invertin, which was found in the serum and caused the better utilization of injected sucrose, came from the intestine. Abderhalden and Brahm also believed that they could induce a production of invertin in the serum of dogs. This was considered to be an example of protective enzymes, which appear, according to Abderhalden, in serum, after introduction of substances normally foreign to the circulation. Their results were not constant. Subsequently, Abderhalden and Kapfberger stated that when the amount of sucrose injected was smaller, the results became constant. A single injection of 5 to 10 cc. of 10 per cent sucrose solution subcutaneously, or 2 cc. of 5 to 10 per cent solution intravenously was followed by the appearance of invertin in the serum in a short time—sometimes as soon as 15 minutes. The inverting power of the serum

remained for 14 days after the first injection. When this sucrase disappeared, a second injection caused the reappearance of the enzyme for another 14 days. Serum which had this inverting power could also change lactose, but not raffinose (experiments with dogs).

Upon the basis of the investigation of sucrose elimination and respiratory exchange after subcutaneous injection of this sugar, Heilner stated that part of the sucrose injected was oxidized in the animal body, assuming that it was first hydrolyzed by the invertin which was stated by others to appear. Pincussohn and Krause also tried to call forth invertin in the blood by injection of sucrose. In seventeen experiments with dogs and rabbits a positive result was found only once.

Kumagai did not succeed by Abderhalden's method; but after repeated injections of a much larger amount of sucrose, he found invertin constantly in the serum. His results seemed far more conclusive than those of Abderhalden and his collaborators. He stated that sucrose had the properties of an antigen and animals could be actively or passively immunized against it. Furthermore, serum which was active to sucrose could act upon the cleavage products and other kinds of sugar, such as milk sugar and galactose. Dextrose was reported transformed to levulose and from the one or the other of these simple sugars a disaccharide was synthesized. Röhmann and Kumagai believed that the product was lactose. Abderhalden and Wildermuth tried to confirm Kumagai's findings with rabbits. Serum which was active to sucrose was never active to any other kind of sugar. In Abderhalden's earlier experiments with dogs, such serum was also active towards lactose, but later it was reported to be negative in the case of rabbits. Inactivation and activation procedures were also negative. Abderhalden and Wildermuth confirmed the observation that a single injection of a very small amount of sucrose was enough to call forth invertin in serum. Abderhalden and Grigorescu, also working with dogs, obtained less constant results. These investigators ascribed such inconstancy to the variability in the occurrence of sucrase in the intestinal cells. The extreme rapidity of elimination of sucrose in some animals was also believed to account for the failure to provoke appearance of inverting enzyme in the blood. Abderhalden imagined that invertin in the serum has its origin in the intestine, because an animal in which the serum was first negative gave positive results in its action on sucrose after feeding this sugar.

Hogan also injected sucrose and lactose into dogs, but did not succeed in finding the corresponding inverting enzyme in the serum. On the other hand, Lombroso accepts the appearance of invertin in serum. Recently Röhmann, in whose laboratory Kumagai had worked, repeated Kumagai's experiments, but the results were far less constant. He says that the uncertainty which exists in the results of Abderhalden and his collaborators is also seen in his and Kumagai's results.

The question naturally arises whether, if an inverting enzyme is actually introduced into the blood, the utilization of parenterally injected sugar will be improved. La Franca reported that

when invertin is injected into the animal body before sucrose injection, the utilization of the latter is improved. This is interesting, when we compare the reports which insist on the appearance of sucrase with those which state that it is impossible to cause any better utilization of sucrose by repeated injection of this sugar.

It is conceivable that blood constituents may interfere with the action of inverting enzymes. Both Hildebrandt and Kumagai state that serum has no power to alter the activity of invertin. This is not in accord with the statements of Eriksson, and Griffin and Nelson.

In view of the numerous conflicting views reported above, I have, at the suggestion of Professor Lafayette B. Mendel, undertaken further investigations of some of the controverted problems.

The plan of the experiments included a reinvestigation of the extent of utilization of sucrose introduced parenterally, under a variety of conditions. In some cases large or small amounts of sugar were frequently injected to learn whether adaptation occurred or invertin was developed in the blood. In other cases active invertin was actually introduced into the blood stream to ascertain whether the enzyme would circulate so as to promote the utilization of sucrose in any way. The enzymatic activity of the blood serum of the experimental animals was repeatedly investigated.

The Utilization of Sucrose after Parenteral Injection.

Sucrose was repeatedly administered parenterally in adult dogs. They were kept in metabolism cages, the urine being collected in bottles containing toluene. The diet consisted of meat, bread, and salt. To prevent contamination of the urine with feces, bone meal (about 10 gm. a day) was mixed with the food. Water was given freely. The body weight of the dogs was nearly constant or decreased only a little during the experiments. The injections were made subcutaneously, intraperitoneally, or intravenously. For intravenous injection local anesthesia with cocaine was usually used, general anesthesia with ether being required only once (Dog B, Feb. 2).

The strength of the injected sugar solutions, estimated by the polariscope, was 10 per cent. Usually 50 cc. were given at one time. During March 15 to 29, 40 to 50 cc. of 40 per cent solution were employed for the injections in Dogs B and D.

The determination of sucrose in the urine was made by the polariscope with readings in Ventzke degrees, mercuric acetate being used to remove the coloring matters and levorotatory substances in the normal urine. 15 cc. of the saturated solution of mercuric acetate for 30 cc. of the urine were enough to gain practically the zero reading at the usual urine concentration. A known amount of sucrose added to the normal urine could be quantitatively determined by this method. At first both mercuric acetate and phosphotungstic acid were used successively, as Neuberg and Ishida suggested for removing protein cleavage products. Afterwards, mercuric acetate alone was found to be satisfactory, if the amount added was not too small and the mixture was not filtered too soon. Benedict's test was used for reducing sugar, Heller's test for protein.

Five invertin preparations were employed. Invertin Solutions I to IV were prepared from compressed yeast, but Invertin V was made from brewer's yeast. Following Hudson (1914), the yeast was washed and mixed with water and toluene in the proportion of 1 gm. yeast: 1 cc. water: 0.06 cc. toluene. It was allowed to autolyze 10 to 14 days. Protein was removed by neutral lead acetate and the excess of lead was removed by hydrogen sulfide. The filtrate was dialyzed for 2 days. The invertin solution was preserved with chloroform. The solution was clear, slightly yellow, and neutral in reaction. Only a very slight turbidity, if any, occurred on heating or adding nitric acid. The solution had no reducing power, but a very strong inverting one. The activity was tested before each trial. In addition, quantitative tests were made with each preparation. To 10 cc. of sucrose solution 1 to 2 cc. of invertin solution were added. In a few experiments the effect of addition of acid or alkali to the mixture was tested. The samples were kept at 37–40°C. for 5 to 20 minutes. Dilute ammonia was then added to stop the action of the invertin and complete the multirotation of the invert sugar (Hudson, 1909). The optical rotation (Ventzke scale) and the reducing power (Benedict's test) were then ascertained. The results of the tests of the invertin preparations are summarized in Table I.

Table II exhibits the numerous data on the utilization of sucrose in dogs after repeated injections of the sugar either alone or with simultaneous or previous injections of active invertin.

TABLE I.

Activity of the Invertin Preparations.

Invertin.	Date.	Sucrose.		Invertin.	Reaction.	Time in thermostat.	Rotation.	Reduction.
		cc.	*per cent*	*cc.*		*min.*	*V°.*	
I.	Jan. 26	25	10	1	Neutral.	5	+29.90	+++
		"	"	1 (Boiled.)	"	5	+36.83	−
II.	Jan. 29	25	10	2	Neutral.	5	+30.85	+++
		"	"	2	"	10	+26.00	+++
		"	"	2 (Boiled.)	"	10	+35.25	−
	Feb. 16	25	10	2	"	20	+26.95	+++
		"	"	2 (Boiled.)	"	20	+35.15	−
		"	"	0 (2 cc. water.)	"	20	+35.10	−
III.	Feb. 7	25	10	2	Neutral.	5	+29.95	+++
		"	"	2 (Boiled.)	"	5	+35.70	−
	" 7	25	0.5	1	"	5	+1.65	+++
		"	"	1	Acid.*	5	+1.38	+++
		"	"	1	Alkaline.†	5	+1.86	−
		"	"	1 (Boiled.)	Neutral.	5	+1.90	−
	" 7	25	0.5	1	"	20	+1.13	+++
		"	"	1	Acid.*	20	+0.59	+++
		"	"	1	Alkaline.†	20	+1.89	−
		"	"	1 (Boiled.)	Neutral.	20	+1.88	−
IV.	Feb. 21	25	0.5	1	Neutral.	10	+1.70	+++
		"	"	1 (Boiled.)	"	10	+2.10	−
V.	Apr. 14	25	0.5	1	Neutral.	5	+1.05	+++
		"	"	1	Acid.*	5	+0.63	+++
		"	"	1 (Boiled.)	Neutral.	5	+1.85	−
	" 14	25	0.5	1	"	20	+0.08	+++
		"	"	1	Acid.*	20	−0.40	+++
		"	"	1 (Boiled.)	Neutral.	20	+1.83	−

* Dilute acetic acid was added.

† Dilute ammonia was added.

TABLE II.

Utilization of Sucrose Injected Parenterally.

	Date.	Sucrose injection.			Invertin injection.			Urine.								
		Time.	Amount.	Place.	Time.	Volume.	Place.	Date.	Time.	Volume.	Sp. gr.	Reaction (litmus).	Sucrose.		Total sucrose recovered.	
			gm.			*cc.*				*cc.*	*1.0 —*		*per cent*	*gm.*	*gm.*	*per cent*
Dog A, ♀ 12.5 kg.	Jan. 19	11.20 a.m.	4.97	Peritoneal cavity.				Jan. 19	12 a.m.	55.0		Acid.	1.02	0.56	4.02	80.9
								" 20	9 "	136.0	54	Alkaline.	2.52	3.43		
								" 20	1 p.m.	61.0	36	Acid.	0.05	0.03		
								" 21	9 a.m.	131.0	36	Alkaline.	0	0		
	" 24	10.20 a.m.	4.97	" "				" 24	10 "	64.0	46	Acid.	0	0	4.04	81.4
								" 25	9 "	71.0	62	"	5.70	4.04		
								" 26	8 "	47.0	48	"	0	0		
	" 26	12.20 a.m.	5.00	" "	10.40 a.m.	5.0 (I)	Subcutaneous.	" 27	9 "	64.0	54	"	6.18	3.96	3.96	79.1
					12.20 p.m.	5.0 (I)	"	" 27	2 p.m.	45.0	44	Neutral.	0	0		
Dog B, ♀ 13.8 kg.	Jan. 24	10.10 a.m.	4.97	Peritoneal cavity.				Jan. 24	11 a.m.	221.0	42	Acid.	0.15	0.32	3.66	73.7
								" 25	9 "	157.0	36	Alkaline.	2.13	3.34		
								" 26	10 "	237.5	36	Acid.	0	0		
	" 26	12.30 p.m.	5.00	" "				" 27	9 "	160.0	48	"	2.32	3.71	3.71	74.1
								" 28	11 "	310.0	38	"	0	0		
	" 28	2.00 p.m.	4.97	" "	12.30 p.m.	5.0 (II)	Subcutaneous.	" 29	9 "	248.0	42	"	1.63	4.05	4.05	81.4
					2.00 "	5.0 (II)	"	" 29	2 p.m.	86.5	40	"	0	0		

Dog B, ♀ 13.8 kg.

Jan. 31	10.00 a.m.	4.97	Peritoneal cavity.	9.00 a.m. 10.00 " 11.00 "	10.0 (II) 10.0 (II) 10.0 (II)	Subcutaneous. " "	Feb. 1	9 a.m.	194.0	37	Acid.	1.72	3.33	3.33	67.0
							" 1	4 p.m.	204.0	18	"	0	0		
Feb. 2	11.10 a.m.	4.98	" "	11.00 "	19.0 (II)	V. jugularis ext.	" 3	9 a.m.	302.0	27	"	1.03	3.10	3.19	64.0
				12.00 "	25.0 (II)	Subcutaneous.	" 3	8 p.m.	147.0	22	"	0.06	0.09		
							" 4	9 a.m.	220.0	26	Neutral.	0	0		
" 4	5.40 p.m.	4.98	" "	5.30 p.m.	40.5 (III)	V. jugularis ext.	" 5	9 a.m.	196.0	25	Neutral.	1.57	3.07	3.07	63.0
							" 5	5 p.m.	163.0	20	Acid.	0	0		
							" 6	10 a.m.	180.0	26	"	0	0		
" 7	11.50 a.m.	4.97	" "				" 8	9 "	300.0	23	"	1.095	3.29	3.29	66.1
							" 8	3 p.m.	49.0	20	"	0	0		
							" 8	5 "	45.0	17	"	0	0		
" 8	6.00 p.m.	4.97	" "				" 9	9 a.m.	171.0	29	"	1.85	3.16	3.44	69.2
							" 9	11 "	63.0	26	"	0.45	0.28		
							" 10	9 "	110.0	27	"	0	0		
" 11	3.00 p.m.	4.97	" "				" 12	9 "	255.0	30	"	1.30	3.32	3.32	66.8
							" 13	10 "	203.0	36	"	0	0		
" 14	12.50 p.m.	5.00	" "				" 14	7 p.m.	129.0	32	"	3.03	3.91	3.91	78.2
							" 15	9 a.m.	56.0	49	"	0	0		
" 16	4.05 p.m.	5.00	V. jugularis ext.	4.00 p.m.	45.0 (II) (Boiled.)	V. jugularis ext.	" 17	9 "	150.0	38	"	2.59	3.98	3.98	79.6
							" 18*	9 "	215.0	36	Neutral.	0	0		

* Protein ±.

TABLE II—*Continued.*

	Date.	Sucrose injection.			Invertin injection.			Urine.								
		Time.	Amount.	Place.	Time.	Volume.	Place.	Date.	Time.	Volume.	Sp. gr.	Reaction (litmus).	Sucrose.		Total sucrose recovered.	
			gm.			*cc.*				*cc.*	*1.0 —*		*per cent*	*gm.*	*gm.*	*per cent*
Dog B, ♀ 13.8 kg.	Feb. 18	3.30 p.m.	5.00	V. jugularis ext.	3.20 p.m.	45.0 (IV)	V. jugularis ext.	Feb. 19*	9 a.m.	219.0	27	Alkaline.	1.51	3.30	3.30	66.0
								" 19	11 "	93.5	40	Acid.	0	0		
	" 19	3.00 p.m.	5.00	" " "	2.50 "	45.0 (IV)	" " "	" 20	11 "	116.0	40	"	1.94	3.21	3.21	64.2
								" 20	12 "	145.0	30	"	0	0		
	" 22	12.00 a.m.	5.00	V. saphena mag.	11.50 a.m.	100.0 (IV)	V. saphena mag.	" 23	9 "	149.0	38	Alkaline.	1.40	2.09	2.09	41.8
								" 24	9 "	499.0	26	Acid.	0	0		
	Mar. 1	1.00 p.m.	5.00	Peritoneal cavity.				Mar. 2†	9 "	207.0	41	"	1.48	3.06	3.06	61.2
								" 3‡	10 "	135.0	45	"	0	0		
	" 15–29		20.0 8 times.	" "				" 15–30§								
	" 30	5.00 p.m.	5.00	" "				" 31	10 a.m.	314.5	42	"	1.34	4.19	4.19	83.8
								Apr. 1	3 p.m.	200.0	24	"	0	0		
	Apr. 3	11.30 a.m.	5.00	" "				" 4	12 a.m.	263.0	44	Neutral.	1.57	4.12	4.12	82.4
								" 5	12 "	378.0	27	Acid.	0	0		

Dog C, ♀ 5.9 kg.

Jan. 31	9.30 a.m.	4.97	Peritoneal cavity.				Feb. 1	9 a.m.	112.0	48	Acid.	3.17	3.55	3.55	71.4
							" 2†‡	9 "	67.0	42	"	0	0		
Feb. 2	12.30 p.m.	4.98	" "	11.30 a.m.	20.0 (II)	Subcutaneous.	" 3	5 p.m.	98.0	50	"	3.57	3.50	3.50	70.2
				12.20 p.m.	20.0 (II)	"	" 4	9 a.m.	90.5	45	"	0	0		
" 4	7.45 p.m.	4.98	" "	6.30 "	55.0 (III)	"	" 5	12 "	162.0	33	Neutral.	2.44	3.95	3.95	79.3
							" 6	10 "	95.0	36	Alkaline.	0	0		
" 7	11.35 a.m.	4.97	V. jugularis ext.	11.30 a.m.	45.0 (III)	V. jugularis ext.	" 8	9 "	191.0	28	Acid.	0.79	1.52	1.52	30.5
							" 8	7 p.m.	90.0	37	"	0	0		
							" 9	9 a.m.	67.0	36	Alkaline.	0	0		
" 9	12.05 p.m.	4.97	" " "	12.00 "	45.0 (III)	" " "	" 9	5 p.m.	122.0	30	Acid.	1.37	1.68	1.68	33.7
							" 10	9 a.m.	70.5	42	Alkaline.	0	0		
" 11	2.00 p.m.	4.97	Peritoneal cavity.				" 12	9 "	100.0	44	Neutral.	3.60	3.60	3.60	72.3
							" 13	10 "	184.0	37	Acid.	0	0		
" 14	1.50 p.m.	1.00	" "												
	3.20 p.m.	1.00	" "												
	4.50 p.m.	1.00	" "				" 15†‡	9 "	66.0	66	"	5.67	3.74	3.74	74.4
							" 15	12 "	74.0	46	"	0	0		
	5.50 p.m.	1.00	" "												
	6.50 p.m.	1.00	" "												

* Protein ±.
† Reduction +.
‡ Reduction ±.
§ Sometimes reduction +.

TABLE II—*Concluded.*

Dog D, ♀ 7.5 kg.

Date.	Sucrose injection.			Invertin injection.			Urine.								
	Time.	Amount.	Place.	Time.	Volume.	Place.	Date.	Time.	Volume.	Sp. gr.	Reaction (litmus).	Sucrose.		Total sucrose recovered.	
		gm.			*cc.*				*cc.*	*1.0 —*		*per cent*	*gm.*	*gm.*	*per cent*
Jan. 31	9.40 a.m.	4.97	Peritoneal cavity.				Feb. 1*	9 a.m.	215.0	48	Acid.	1.80	3.88	3.88	78.0
							" 3*	9 "	246.0	42	"	0	0		
Feb. 9	12.35 p.m.	4.97	V. jugularis ext.	12.30 p.m.	45.0 (III)	V. jugularis ext.	" 9	7 p.m.	140.0	18	Alkaline.	0.82	1.15	1.15	23.1
							" 10	2 "	168.0	32	Acid.	0	0		
" 11	2.30 p.m.	4.97	" " "				" 12	9 a.m.	175.0	29	Alkaline.	2.35	4.11	4.11	82.1
							" 13	10 "	180.0	40	Acid.	0	0		
" 25	12.10 p.m.	5.00	Peritoneal cavity.	11.50 a.m.	45.0 (III) (Boiled.)	Peritoneal cavity.	" 26	9 "	273.0	34	Neutral.	1.60	4.39	4.39	87.8
							" 26	5 p.m.	131.0	17	Acid.	0	0		
Mar. 2	7.00 p.m.	4.91	Subcutaneous.				Mar. 3*	9 a.m.	79.5	48	"	3.90	3.10	3.56	72.5
							" 4	9 "	65.0	50	Neutral.	0.70	0.46		
							" 4	5 p.m.	120.0	31	Acid.	0	0		
" 15–29		16.0‡ 18.0‡	Peritoneal cavity.				" 15–30†								
" 30	4.50 p.m.	5.00	" "				" 31	4 p.m.	273.5	30	"	1.62	4.42	4.42	88.4
							Apr. 2	12 a.m.	123.0	32	"	0	0		
Apr. 3	12.00 a.m.	5.00	" "				" 4	12 "	230.0	39	"	1.92	4.41	4.41	88.2
							" 5	12 "	145.0	32	"	0	0		
" 6	11.00 a.m.	5.00	" "	10.55 a.m.	90.0 (V)	V. brachialis.	" 7*	11 "	237.0	40	Alkaline.	1.33	3.15	3.15	63.0
							" 8	12 "	170.0	45	Neutral.	0	0		

Dog E, ♂ 12.1 kg.	Mar. 30	4.40 p.m.	5.00	Peritoneal cavity.				Apr. 1	11 a.m.	215.0	35	Acid.	1.39	2.99	3.22	64.4
								" 2	11 "	100.0	33	"	0.24	0.23		
								" 3	11 "	137.0	45	Neutral.	0	0		
	Apr. 3	12.00 a.m.	5.00	" "				" 5*	9 "	323.0	48	Acid.	1.17	3.79	3.79	75.8
								" 6	12 "	192.0	51	Neutral.	0	0		

* Reduction ±.

† Sometimes reduction +.

‡ 4 times.

Injection of Sucrose Alone.

A résumé of the essential features with respect to the repetition of the sugar injections and their effect on the utilization is given in Tables III and IV.

TABLE III.

Data Regarding Injections of Sucrose and Invertin.

Dog.	Duration of experiment.	Injection of sucrose alone.		Injection of sucrose and invertin.	
		Sucrose per kg. body weight.	No. of injections.	Sucrose per kg. body weight.	No. of injections.
	days	*gm.*		*gm.*	
A	6	0.4	2	0.4	1
B	70	0.4 1.5	9 8	0.4	8
C	15	0.9	3	0.9	4
D	67	0.7 2.1 2.4	5 4 4	0.7	3
E	5	0.4	2		

TABLE IV.

Recovery of Sucrose (in Urine) after Repeated Injections (Sucrose Alone Injected).

Dog.	Maximum.	Minimum.	Average.	After first injection.	After last injection.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
A	81.4	80.9	81.2	80.9	81.4
B	83.8	61.2	72.9	73.7	82.4
C	74.4	71.4	72.7	71.4	74.4
D	88.4	72.5	81.8	78.0	88.2
E	75.8	64.4	70.1	64.4	75.8

Average of 21 injections = 75.5 per cent.

The tables show clearly that the parenteral utilization of sucrose was not improved by the repeated injections. This agrees with the reports of Mendel and Kleiner, and Hogan. Elimination of sucrose usually ends within 24 hours after the injection. The same degree of utilization of sucrose occurred after the different paths—intraperitoneal, intravenous, or subcutaneous—of administration. There was no evidence of interference with the normal renal function. Usually the volume of the urine was undiminished and protein did not appear. When the amount of sugar injected was 0.4 to 0.9 gm. per kilo of body weight, more than a trace of reducing substance was never found. With larger amounts of sugar (1.5 to 2.4 gm. per kilo of body weight) a detectable amount of reducing sugar was sometimes found in the urine. The general condition of the dogs was not affected after sugar injections, except for a slight transitory fever after two or three of the injections.

Injection of Invertin and Sucrose.

Invertin and sucrose were injected in succession, in sixteen experiments, as shown by the protocols recorded in Table II. A better utilization of sucrose, particularly after intravenous injections, was gained as follows:

Dog.	Invertin injected.	Sugar recovered.
	cc.	*per cent*
B.....	100 (IV)	41.8
C.....	45 (III)	30.5
C.....	45 (III)	33.7
D.....	45 (III)	23.1

A large amount of invertin was required to produce noteworthy effects. When 40.5 cc. (III) to 45.0 cc. (IV) of invertin were injected in Dog B, 63 to 66 per cent of sucrose was recovered. These values are scarcely lower than that sometimes obtained without the invertin injection. When invertin and sucrose were administered by different paths—*i.e.*, one intravenously, the other intraperitoneally—the utilization was only a little better than in the control without invertin, or else no difference at all was seen.

The protocols in general indicate that the enzyme of yeast is able to invert sucrose in the circulation. To produce such a result the enzyme and its substrate must, however, be brought together in the organism. For this reason, the utilization is improved when the enzyme is introduced directly and almost simultaneously into the circulation with the sugar; but after subcutaneous administration of invertin, whereby it reaches the rapidly circulating sugar slowly, if at all, little advantage is noted. Even when an improved utilization was noted, reducing sugar was not found in the urine. La Franca reported much better utilization than was found in my experiments. In his paper there is no description of the activity of the invertin he used.

On the 3rd day after the intravenous injection of invertin the utilization of sucrose was the same as before the injection of the invertin. (Dog C, 33.7 per cent on Feb. 9, but 72.3 per cent on Feb. 11; Dog D, 23.1 per cent on Feb. 9, but 82.1 per cent on Feb. 11.) This agrees with the result that the serum taken 20 to 24 hours after the intravenous injection of invertin no longer shows an inverting power (compare Tables VIII and IX). It is evident, therefore, that the inverting power after injection of invertin is not long retained.

Does the Blood Acquire Inverting Power after Parenteral Injection of Sucrose?

Serum was examined by the polariscope, before and after incubating with sucrose. A 2.5 cm. tube was used when the amount of serum was small; and a 2 dm. tube, when more serum was available. Blood was taken by venopuncture. After 1 or 2 hours serum was separated by centrifugation. For the 2.5 cm. tube 1 cc. serum + 1 cc. 10 per cent sucrose + 2 cc. water + 1 drop of toluene were used. This mixture, in tightly stoppered tubes, was kept 40 to 48 hours at 37–40°C. The protein was removed by the colloidal iron method (1 cc. colloidal iron and 0.1 gm. powdered Na_2SO_4) before the filtrate was examined with the polariscope. For this series the final volume represented a dilution of the serum to five times the original. For the 2 dm. tube, 3 cc. serum + 3 cc. 10 per cent sucrose + 6 cc. water

+ 3 drops of toluene were kept in the thermostat under the same conditions. Then 3 cc. colloidal iron, 0.3 gm. Na_2SO_4, and 15 cc. water were added. The final volume was ten times that of the original serum. An attempt was also made to "activate" the invertin by making the solution neutral or very slightly acid, by addition of acetic acid of about 0.025 N and 0.013 N strength. In the tables, the different degrees of acidity are designated as "acid (stronger)" and "acid (weaker)." The procedure, when acid was added, was as follows: 1 cc. serum + 1 cc. 10 per cent sucrose + 1 cc. acid (weaker) + 1 cc. water + 1 drop of toluene (for the 2.5 cm. tube), or 3 cc. serum + 3 cc. 10 per cent sucrose + 3 cc. acid (stronger) + 3 cc. water + 3 drops of toluene (for the 2 dm. tube). The reaction of the mixture was neutral, or very slightly acid to litmus. As a control, the same mixture was examined at once without incubation. When 0.9 per cent NaCl was used instead of serum, sometimes a very slight decrease of rotation and reducing substance were noted after incubating. When the same acid was added to serum, the change, which was due to the acid alone, never appeared. Rotations are expressed in degrees of the Ventzke scale. In numerous preliminary trials, it was shown that the removal of protein by colloidal iron did not interfere with the determination of sucrose and its digestion products. The experimental error in these trials was not greater than 0.1°V. Reducing power was tested by Benedict's method, and controlled by comparison with the normal serum which contains a small amount of dextrose. The test was made by adding three or six drops (according as the dilution of the serum was five or ten times) to 5 cc. of the reagent. These proportions gave no visible reduction with normal serum. The dogs used had served in the experiments recorded in Table II. The results are summarized in Table V.

Five *rabbits* were also used. Sucrose was always injected in 40 per cent solution.

Rabbit A, used as a *control* without injection.

Rabbit B, 2.3 kg. *Sucrose* was injected *subcutaneously; viz.*, 4 gm. on Mar. 17, 6 gm. on Mar. 18, 20, and 22, 8 gm. on Mar. 25, 27, and 29, 16 gm. on Apr. 2.

Rabbit C, 2.6 kg. *Sucrose* was injected *intraperitoneally; viz.*, 6 gm. on Mar. 18, 20, and 22, 8 gm. on Mar. 25, 27, 29, and Apr. 2.

TABLE V.

Study of Inverting Power of Serum after Parenteral Injection of Sucrose (Dogs).

Dog.	Sugar injection before serum examination.	Date of serum examination.		Examined immediately.						Examined after incubating 40 to 48 hrs. at 37–40°C.					
				Serum + sucrose.			0.9% NaCl + sucrose.			Serum + sucrose.			0.9% NaCl + sucrose.		
				Alone.	+Acid (weaker).	+Acid (stronger).	Alone.	+Acid (weaker).	+Acid (stronger).	Alone.	+Acid (weaker).	+Acid (stronger).	Alone.	+Acid (weaker).	+Acid (stronger).
B	5 gm. 15 times. Jan. 24–Mar. 1	Mar. 10	Rotation V°	+0.80		+0.78				+0.85		+0.79			
			Reduction	−		−				−		−			
	"	" 11	Rotation V°	+0.81		+0.80				+0.80		+0.81	+0.78		+0.70
			Reduction	−		−				−		−	−		+
	20 gm. 8 times. Mar. 15–29	" 30	Rotation V°	+0.83	+0.83				+0.80	+0.78	+0.78			+0.74	
			Reduction	−	−				−	−	−			+	
C*	5 gm. 7 times. Jan. 31–Feb. 14	Feb. 29	Rotation V°	+3.85†						+3.80					
			Reduction	−						−					
D	5 gm. 4 times. Jan. 31–Feb. 25	Mar. 2	Rotation V°	+0.88						+0.83					
			Reduction	−						−					
	5 gm. Once. Mar. 2	" 7	Rotation V°	+0.78			+0.75			+0.80		+0.79			+0.76
			Reduction	−			−			−		−			±
	16 gm. 4 times. 18 gm. 4 times. Mar. 15–29	" 30	Rotation V°	+0.85	+0.85			+0.80		+0.78	+0.78			+0.75	
			Reduction	−	−			−		−	−			+	
	5 gm. Once. Apr. 3	Apr. 6	Rotation V°	+3.80†						+3.80		+3.80			
			Reduction	−						−		−			
	5 gm. Once. Apr. 6	" 12	Rotation V°	+3.83†						+3.80	+3.80	+3.78			
			Reduction	−						−	−	−			

* Dog C died of pneumonia and severe hemorrhagic enteritis. Blood was taken from the heart after death. No invertin was found in the mucous membrane of the intestine.

† These were examined with 2 dm. tubes, all the others with 2.5 cm. tubes.

TABLE VI.

Study of Inverting Power of Serum after Parenteral Injection of Sucrose (Rabbits).

Rabbit.	Date of serum examination.		Examined immediately.			Examined after incubating 40 to 48 hrs. at 37–40° C.				
			Serum + sucrose.		0.9 per cent NaCl + sucrose + acid (stronger).	Serum + sucrose.			0.9 per cent NaCl + sucrose.	
			Alone.	+ Acid (stronger).		Alone.	+ Acid (weaker).	+ Acid (stronger).	+ Acid (weaker).	+ Acid (stronger).
A	Feb. 15.....	Rotation V°	+0.85			+0.86				
		Reduction	−			−				
B	Apr. 4.....	Rotation V°	+0.85			+0.83	+0.83	+0.80	+0.70	
		Reduction	−			−	−	−	+	
C	Apr. 4.....	Rotation V°	+0.88				+0.83			
		Reduction	−				−			
	" 4.....	Rotation V°	+3.85*			+3.85	+3.83		+3.20	
		Reduction	−			−	−		+	
D	Mar. 31.....	Rotation V°	+0.85	+0.80	+0.83	+0.80	+0.80	+0.80	+0.68	+0.68
		Reduction	−	−	−	−	−	−	+	+
E	Apr. 1.....	Rotation V°	+0.80	+0.83	+0.80	+0.80	+0.80	+0.80	+0.70	+0.68
		Reduction	−	−	−	−	−	−	±	+
	" 5.....	Rotation V°	+3.83*			+3.80	+3.80			
		Reduction	−			−	−			

* These were examined with 2 dm. tubes, all the others with 2.5 cm. tubes.

Rabbit D, 2.5 kg. *Sucrose* was injected *intraperitoneally; viz.*, 4 gm. on Mar. 17, 6 gm. on Mar. 18, 20, and 22, 8 gm. on Mar. 25, 27, and 29.

Rabbit E, 2.8 kg. *Sucrose* was injected *intravenously; viz.*, 4 gm. on Mar. 17, 6 gm. on Mar. 18, 20, and 22, 8 gm. on Mar. 25, 27, 29, and Apr. 2.

Bread, corn, carrots, and cabbage were fed. The sucrose injection caused no noticeable disturbance. The urine sometimes contained a small amount of reducing sugar, but usually none. The records are given in Table VI.

The records of Tables V and VI show that the serum had no power to decrease the rotation of serum-sucrose mixtures; *i.e.*, to invert sucrose. The dogs here used were the same ones, in which invertin was injected. It is scarcely to be assumed that previous injection of yeast invertin prevented the appearance of a "protective" sucrase.

Only a few experiments were made to see if the serum, which is taken after sucrose injection, has also the power to change dextrose into levulose and synthesize lactose from these. The method was the same as above mentioned (with the 2.5 cm. tube). 10 per cent dextrose solution and Seliwanoff's test were used instead of sucrose solution and Benedict's test. The results were negative (see Table VII).

TABLE VII.

Action of Serum upon Dextrose.

Animal.		Date.	Examined immediately.		Examined after incubating 40 to 48 hrs. at 37–40° C.	
			Rotation.	Seliwanoff's test.	Rotation.	Seliwanoff's test.
			V°.		*V°.*	
Dog.	B	Mar. 30	+0.63	–	+0.63	–
	D	" 30	+0.63	–	+0.63	–
Rabbit.	B	Mar. 31	+0.63	–	+0.63	–
		Apr. 2	+0.70	–	+0.60	–
	C	" 2	+0.70	–	+0.63	–
	D	Mar. 31	+0.63	–	+0.63	–
	E	Apr. 1	+0.65	–	+0.63	–

Examination of the Blood Serum and Urine for Invertin after Injection of Yeast Invertin.

The inverting power of the serum taken at various intervals after invertin injection was examined by the method already described (2.5 cm. tubes).

Urine was collected during 48 hours after the invertin injections, and examined for invertin, as follows: 20 cc. urine + 10 cc. 10 per cent sucrose + 1 cc. water + 3 drops of toluene were incubated 24 hours at 37–40°C. To 25 cc. of the mixture 10 cc. of the mercuric acetate solution were added. The filtrate was then examined polariscopically. As a control, boiled urine or water was used instead of urine; and by adding 1 cc. of invertin instead of 1 cc. of water it was demonstrated that if invertin was present in the urine, it could be found by this method. All portions of the urine were acid, except the second sample from Dog D. In some experiments two drops of dilute acetic acid were added to increase the activity of invertin, if any was present. The results are given in Tables VIII and IX.

TABLE VIII.

Dog. B. Mar. 10, 100 Cc. Invertin (IV) Were Injected into V. Femoralis. Serum Tests.

Serum taken.		Examined immediately.		Examined after incubating 40 hrs. at 37–40° C.			
		Serum + sucrose.		Serum + sucrose.		0.9 per cent NaCl + sucrose.	
		Alone.	+ Acid (stronger).	Alone.	+ Acid (stronger).	Alone.	+ Acid (stronger).
Just before injection.	Rotation V°	+0.80	+0.78	+0.85	+0.79		
	Reduction	−	−	−	−		
10 minutes after injection.	Rotation V°	+0.80	+0.79	+0.56	−0.28	+0.80	+0.75
	Reduction	−	±	+	+++	−	+
1 hour after injection.	Rotation V°	+0.85	+0.83	+0.73	−0.04		
	Reduction	−	−	±	+++		
20 hours after injection.	Rotation V°	+0.81	+0.80	+0.80	+0.81	+0.78	+0.70
	Reduction	−	−	−	−	−	+

TABLE VIII—*Concluded.*

Urine Tests.

Urine of		Urine + sucrose.					Water + sucrose + invertin (IV).
		Alone.	Alone (boiled urine).	+ Acid.	+ Acid (boiled urine).	+ Invertin (IV).	
1st day.	Rotation V°	+8.55	+8.48	+8.50	+8.50	−1.60	−1.80
	Reduction	−	−	−	−	+++	+++
2nd day.	Rotation V°	+8.70	+8.70	+8.77	+8.70	+6.80	−2.65
	Reduction	−	−	−	−	+++	+++

TABLE IX.

Dog. D. Mar. 6, 45 Cc. Invertin (IV) Were Injected into V. Femoralis.

Serum Tests.

Serum taken.		Examined immediately.		Examined after incubating 40 hrs. at 37–40° C.			
		Serum + sucrose alone.	0.9 per cent NaCl + sucrose alone.	Serum + sucrose.		0.9 per cent NaCl + sucrose.	
				Alone.	+ Acid (stronger).	Alone.	+ Acid (stronger).
10 minutes after injection.	Rotation V°	+0.78	+0.80	+0.63	−0.16	+0.75	+0.75
	Reduction	±	−	+	+++	−	±
1 hour after injection.	Rotation V°	+0.82	+0.80	+0.69	−0.16		
	Reduction	−	−	+	+++		
24 hours after injection.	Rotation V°	+0.78	+0.75	+0.80	+0.79		+0.76
	Reduction	−	−	−	−		±

Urine Tests.

Urine of 48 hrs. after injection.		Urine + sucrose.					Water + sucrose + invertin (IV).
		Alone.	Alone (boiled urine).	+ Acid.	+ Acid (boiled urine).	+ Invertin (IV).	
1	Rotation V°	+8.80	+8.80	+8.80	+8.80	+1.90	−2.60
	Reduction	−	−	−	−	+++	+++
2*	Rotation V°			+8.73	+8.73	+5.15	+1.80
	Reduction			−	−	+++	+++
3	Rotation V°	+8.78	+8.75	+8.73	+8.75	−0.70	−2.15
	Reduction	−	−	−	−	+++	+++

* As this portion was alkaline, two drops of acetic acid were always added.

These experiments indicate that the serum taken 10 minutes or 1 hour after the end of the invertin injection had an inverting power. When dilute acid was added, this power increased markedly, but it was no longer present in the serum collected 20 to 24 hours after injection. Invertin was not eliminated in the urine.

The Effect of Serum or Blood upon the Activity of Yeast or Intestinal Invertin (in Vitro).

It is conceivable that blood may interfere in some way with the demonstration of the presence of sucrase. Accordingly this problem was investigated. 10 cc. of defibrinated pig's blood (or as a control 0.9 per cent NaCl) + 2 cc. yeast invertin (IV) (as a control, boiled invertin or 0.9 per cent NaCl) + 5 cc. 10 per cent sucrose were incubated 40 minutes at 37–40°C. 53 cc. of distilled water, 30 cc. of colloidal iron, and 0.5 gm. of powdered Na_2SO_4 were added successively. To 30 cc. of the filtrate 1 cc. of colloidal iron was added again. After addition of dilute ammonia to 25 cc. of the filtrate the rotation and reducing power were tested (2 dm. tubes). Invertin and sucrose were added either together or successively after an interval (see Table X).

The results show that, though the invertin could act in defibrinated pig's blood, its efficiency was decreased markedly. When invertin and blood were kept together a little while before the sucrose addition, the decrease of the activity of the invertin seemed to be larger.

In another series (Table XI), the activity of yeast invertin (IV) added to normal dog serum was examined by the same method as that used in the investigation of the serum after sucrose or invertin injections (p. 534). The 2.5 cm. tubes were used. Two invertin solutions, diluted ten and twenty times, were prepared. In the table one is described as "invertin (stronger)," the other as "invertin (weaker)." The mixing was done as follows: 1 cc. serum + 1 cc. 10 per cent sucrose + 1 cc. acid (stronger) + 1 cc. invertin (weaker) + 1 drop of toluene. For analysis immediately or after incubation 1 cc. of colloidal iron and 0.1 gm. of powdered Na_2SO_4 were added.

TABLE X.

Activity of Invertin in Defibrinated Pig's Blood.

	Blood + sucrose.			0.9 per cent NaCl + sucrose + invertin (2 cc.).	The addition of the invertin and sucrose was made at the same time.
	+ Invertin (2 cc.).	+ Boiled invertin (2 cc.).	Alone.		
Rotation V°	−0.05	+1.95	+1.95	−0.45	
Reduction	+++	−	−	+++	

	Blood + sucrose.			0.9 per cent NaCl + sucrose.		The invertin was added and the mixture was kept 15 minutes in the thermostat before the sucrose addition.
	Alone.	+ Invertin (2 cc.).	+ Invertin (1 cc.).	+ Invertin (2 cc.).	+ Invertin (1 cc.).	
Rotation V°	+1.93	+0.80	+1.30	−0.48	+0.05	
Reduction	−	+++	+++	+++	+++	

	Blood + sucrose.			0.9 per cent NaCl + sucrose + invertin* (2 cc.).
	+ Invertin* (2 cc.).	+ Invertin† (2 cc.).	+ Invertin‡ (2 cc.).	
Rotation V°	+0.50	+0.25	+0.55	−0.45
Reduction	+++	+++	+++	+++

* The invertin was first added. After keeping 15 minutes in the thermostat, the sucrose was added.

† The invertin and sucrose were added at the same time.

‡ The invertin was first added. After keeping 1 hour in room temperature and 15 minutes in the thermostat, the sucrose was added.

To study further the effect of serum upon intestinal sucrase, serum and intestinal extracts, prepared from the same dog (D) by the method described by Mendel and Mitchell (1907), were tested together. The scrapings of the mucous membrane of the whole intestine were extracted for 24 hours with double their volume of water. From this very slightly acid extract, solutions, diluted three and six times respectively, were made—"invertin (stronger)" and "invertin (weaker)."

The results in Table XI show that yeast and intestinal extracts can invert sucrose in the presence of dog serum, although their activity is decreased very much, compared with the control trials without serum. When dilute acid was added the enzymatic reaction increased markedly. In control experiments with 0.9 per

TABLE XI.

Activity of Invertin in Dog Serum.

			Examined immediately.		Examined after incubating 40 hrs. at 37–40°C.								
					+Acid (weaker).		+Acid (stronger).		Without acid.		+Acid (weaker).	+Acid (stronger).	
			Neither acid nor invertin added.	+Acid (stronger) + invertin (stronger).	+Invertin (weaker).	+Invertin (stronger).	+Invertin (weaker).	+Invertin (stronger).	+Invertin (weaker).	+Invertin (stronger).	Without invertin.		Neither acid nor invertin added.
Activity of yeast invertin.	Normal dog serum + sucrose.	Rotation V°	+0.80	+0.75	−0.11	−0.11	−0.14	−0.15	+0.70	+0.63	+0.75	+0.75	+0.75
		Reduction	−	±	+++	+++	+++	+++	+	+	−	−	−
	0.9 per cent NaCl + sucrose.	Rotation V°	+0.78	+0.78	+0.05	−0.03	−0.05	−0.15	0.0	−0.05			
		Reduction	−	±	+++	+++	+++	+++	+++	+++			
	Serum of Dog D + sucrose.	Rotation V°	+3.83	+3.82	+3.08	+2.18	+0.25	−0.75	+3.78	+3.75	+3.88	+3.88	+3.88
		Reduction	−	±	+++	+++	+++	+++	−	±	−	−	−
	0.9 per cent NaCl + sucrose.	Rotation V°			+0.05	−0.58	−0.43	−1.00	+3.13	+2.83			
		Reduction			+++	+++	+++	+++	++	++			
Activity of intestinal invertin of Dog D.	Serum of Dog D + sucrose.	Rotation V°		+3.80	+2.45	+1.35	+0.75	+0.33	+3.45	+2.00			
		Reduction		−	++	+++	+++	+++	++	+++			
	0.9 per cent NaCl + sucrose.	Rotation V°			+3.60	+2.75	+3.63	+3.28	−0.65	−1.00			
		Reduction			±	++	±	+	+++	+++			

cent NaCl, the potency of yeast invertin increased with acid addition, but the activity of intestinal invertin decreased with the same acid addition. Two explanations suggest themselves. (1) The yeast invertin solution was neutral, but the intestinal invertin solution was very slightly acid. Therefore acid addition to the mixture of the physiological solution and intestinal invertin solution caused an augmented acidity, which may have inhibited the activity of the invertin. (2) The optimum point of the reaction of the medium may be different for these two kinds of invertin. It is clear, though, that invertin from both sources can invert sucrose in serum.

SUMMARY AND DISCUSSION.

In correspondence with earlier observations in this laboratory, sucrose, administered parenterally to dogs, was not eliminated quantitatively in the urine. The amount recovered varied considerably, averaging 76 per cent when sucrose was injected in doses of 0.4 to 0.9 gm. per kilo of body weight. The elimination was usually concluded within 24 hours. The degree of utilization was essentially the same for the different paths of parenteral introduction of the sugar.

The apparent utilization of a small part of the sucrose, as judged by its failure to be excreted by the kidneys, may be due to the presence or rapid production of sucrase in the blood. Other explanations, such as the assumption of an excretion of part of the sugar into the intestine and the reabsorption of the products of its inversion there, are discussed above.

The utilization of the sucrose was not essentially changed after many repeated injections of sugar. This likewise is in accord with earlier experience in this laboratory and is not what one would expect if parenteral introduction of a disaccharide leads to any abundant formation of protective enzymes which can hydrolyze it. It is possible, of course, that the actual amount of inverting enzyme brought into the circulation by the parenteral injections is too small to affect the utilization of large amounts of a sugar that can be rapidly eliminated. It is also possible that invertin is produced in considerable amount, but cannot act effectively under the conditions prevailing in the circulation. These questions will be considered below.

Experiments *in vitro* showed that the inverting power of active yeast or intestinal extracts was not lost in the presence of defibrinated blood or serum, although it was considerably decreased. Addition of acid facilitated the invertin reaction and therefore was carried out in some of the experiments with serum.

That the activity of sucrase is retained in the circulation itself was shown by the results of injecting solutions of active yeast invertin; for under such conditions injected sucrose failed to reappear in as large amounts as usual. The better utilization was not due to any failure of the kidney functions in excreting sucrose. The invertin can be demonstrated in the blood after injection of such an enzyme extract. It soon disappears from the circulation, and can no longer be detected in the serum at the end of 20 to 24 hours. The better utilization of sucrose thereupon does not continue to be manifested. Invertin was not excreted into the urine in my experiments.

The inverting power of the blood serum, after intravenous injections of yeast invertin, under the experimental conditions indicated in Tables VIII and IX, is shown by the following decreases in the optical rotation of the sucrose-serum mixtures: V° 0.24, 0.12, 0.15, 0.13 in 40 hours. It is almost impossible to compare the data given by other investigators, owing to the great diversity of conditions and methods employed. From published protocols the following are selected for consideration.

Abderhalden and Kapfberger: 0.5 cc. serum + 0.5 cc. 10 per cent sucrose + 7 cc. 0.9 per cent NaCl. Rotation was examined without removing protein (length of the tube not given). Decrease of rotation was about 0.06°, 0.15°, 0.42°, etc., in 15 to 99 hours.

Abderhalden and Wildermuth: 1 cc. serum + 1 cc. 5 per cent sucrose. Protein was not removed (length of the tube not given). Decrease of rotation was about 0.08°, 0.1°, 0.17°, 0.18°, etc., in 32 to 49 hours.

Kumagai: 2.5 cc. serum + 2.5 cc. 5 per cent sucrose + 10 cc. water. After protein was removed (1 gm. sodium acetate and 1 cc. 30 per cent ferric chloride), rotation was tested with a 1 dm. tube. Decrease of rotation was about 10′, 15′, 42′, 1° 30′, 1° 50′, etc., in 20 to 48 hours.

Röhmann: 0.5 cc. serum + 0.5 cc. 10 per cent sucrose + 9 cc. water. After protein was removed (0.2 to 0.5 gm. sodium acetate and 4 to 5 drops of 30 per cent ferric chloride), rotation was tested with a 1 dm. tube. Decrease of rotation was about 12′, 18′, 20′, 44′, 51′, etc., in 24 to 48 hours.

If such apparent development of invertin were facilitated by parenteral injections of sucrose, one would expect, in the light of my own figures for the serum of animals actually known to contain active invertin, an improved utilization of parenterally introduced sucrose. No quantitative statistics on this point have been published by the European investigators.

Examination of the serum of dogs and rabbits after repeated parenteral injections of sucrose in both small and large doses has uniformly failed, in the present investigations, to demonstrate the presence of invertin in amounts detectable by the methods employed. Glucose was likewise unaltered by the serums of the animals examined.

Some evidence was obtained that the failure to find sucrase in the serum was not due to lack of the enzyme in the intestine, which Abderhalden has assumed to be the source of the blood invertin.

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A COMPARISON OF THE RESULTS OBTAINED BY THE COLORIMETRIC AND GRAVIMETRIC DETERMINATIONS OF CHOLESTEROL.

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It has been observed in the course of a considerable number of cholesterol determinations made by the colorimetric method of Autenrieth and Funk,[1] and the digitonin method of Windaus[2] (gravimetric) that the averages of values obtained were considerably higher in the colorimetric than in the gravimetric analyses. When this fact became appreciated, check determinations were undertaken on the same specimens of blood, and it soon was evident that there was a real source of error in one of the methods, since there was invariably a difference of as much as 10 per cent and often more, the colorimetric results being higher. In a new modification of the colorimetric determination recently published, Bloor[3] has claimed still higher results than those obtained by the Autenrieth-Funk method, by an average of 20 per cent. Grave doubts were thus cast on the reliability of the digitonin method. Since, however, this is the only available means of determining separately the free and combined forms of cholesterol it seemed highly desirable to undertake a critical study of the two procedures and to attempt to locate, and, if possible, to rectify the error. To this end it was necessary to study both the actual technique of the determination and the method of extraction of the cholesterol from the tissues.

In the literature there is comparatively little which might throw any light upon the question. Autenrieth and Funk[1] quote one analysis in which

[1] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

[2] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.

[3] Bloor, W. R., *J. Biol. Chem.*, 1916, xxiv, 227.

practically identical figures for total cholesterol in the blood were obtained by their colorimetric method and by the digitonin method. For the digitonin method, however, the blood was dried with plaster of Paris, and after grinding, extracted for 6 days in a Soxhlet with ether. Since it is improbable that ether ever brings about a complete extraction of cholesterol from dried blood (see below), it is a little hard to understand how this could have been so. Csonka[4] gives an isolated instance where gravimetric and colorimetric determinations agreed quite well, the blood having been first digested with alcoholic potash before extraction. Weston[5] investigated the gravimetric method and obtained results which were much *too high* (by about 9 per cent) when a weighed amount of cholesterol was added to blood, and the latter then extracted by the method devised by him.

The colorimetric methods, of which there are several modifications, are fairly simple, and if accurate, should be of considerable value where nothing more than a determination of total cholesterol is required. It is true that quantitative methods involving colorimetry are criticized by many as introducing a considerable degree of personal error. The green color, however, which is produced in the Liebermann-Burchard test for cholesterol is a most satisfactory one to measure with a colorimeter, particularly if from 1 to 4 mg. of cholesterol are used. Even in those modifications where only 0.3 to 0.5 mg. are used, the color can be compared with a fair degree of accuracy, though not so easily as with the larger amounts. It thus seems desirable to carry out the colorimetric test with the entire extract of the 2 or 3 cc. of blood used, rather than to dilute it to 100 cc. and use a 5 or 10 cc. aliquot.

In order to check the personal error in using a colorimeter with this color, the following experiment was done.

From a stock solution of Merck's cholesterol in chloroform, containing 0.0030 gm. in 5 cc., four unknown solutions were prepared by an associate, using varying quantities of the standard plus enough pure chloroform to bring the volume up to 5 cc. 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid were then added to each tube, and also to a standard tube containing 2.5 cc. of the stock solution and 2.5 cc. of chloroform, and thus 0.0015 gm. of cholesterol. After the color had developed, a

[4] Csonka, F. A., *J. Biol. Chem.*, 1916, xxiv, 431.

[5] Weston, P. G., *J. Med. Research*, 1913–14, xxix, 457.

series of ten readings was made for each unknown solution, using a Duboscq colorimeter, with the standard set at 10 mm., the average taken, and calculations were made. The following results were obtained:

Cholesterol.

Taken. *gm.*	Found. *gm.*
0.00060	0.00056
0.00180	0.00178
0.00240	0.00241
0.00108	0.00086

The agreement in the first three cases was excellent. The fourth tube, on the other hand, shows a rather great error, but it may serve to illustrate a difficulty which is occasionally, though seldom, met with; namely, the development of a green color of a markedly different shade from that produced in most cases. Such a variation appeared in the fourth tube of the above experiment, and undoubtedly accounted for the error. Why this should have occurred in a tube containing only pure cholesterol and chloroform is not clear—possibly from the presence of a little dirt. Autenrieth and Funk[1] state, however, that it is not infrequent to have a green color develop containing a shade of yellow or brown, but that this does not interfere with the values obtained by the colorimeter. That a difference in the character of the color in an unknown and in the standard should cause an error is only natural, and this is illustrated above. Determinations made on blood in this laboratory have only infrequently shown this variation in color. It is possible that when present, it results from the use, without proper decolorization, of a chloroform extract which itself has a more or less marked reddish or yellowish brown color. This is practically always true of the chloroform when it first separates from the alkaline blood mixture. Autenrieth and Funk[1] say this can be removed simply by shaking the chloroform with dry sodium sulfate, which dehydrates and at the same time removes the color from the extract. This has not been uniformly successful in my hands. Csonka[4] has recently published a modification of the method to remedy this defect which involves the use of barium chloride solution in the digestion mixture together with a specially constructed digestion and

extraction flask, by means of which he has obtained good results. A simple and at the same time a most effective way to obtain a perfectly colorless solution is to unite the chloroform extracts and shake them out once or twice with distilled water in a separatory funnel, and then dehydrate with sodium sulfate. The water quickly and completely removes all color from the chloroform.

While the Autenrieth-Funk method has been used throughout as the simplest and most accurate of the colorimetric methods, the method recently described by Bloor[3] has been run parallel with it in several analyses. Bloor's statement that his method gives considerably higher results than the Autenrieth-Funk method has been entirely confirmed, but in the light of what is to follow, it appears that to whatever cause this increase is due, it is not due to cholesterol itself. A possible cause for a part, at least, of the excess value may be found in the slight brownish color invariably present in the chloroform solution of extracts prepared by Bloor's method. The alcohol-ether extraction method itself is most excellent, and has been used with good results in gravimetric determinations, as will appear later, but when an aliquot of this is evaporated, no matter how carefully one attempts to avoid heating the residue after it is dry (which Bloor claims to be the cause of the brown color), and even when it is evaporated before a fan at room temperature, there is still a slight brown tinge to the chloroform solution of the residue. This may be, however, only one factor which raises the value, and other things may play even a more important part.

In order to check the error in the digitonin determination, a few analyses were made on Merck's pure cholesterol. The cholesterol was dissolved in alcohol, brought to boiling, and a 1 per cent solution of digitonin in 90 per cent alcohol added, using 25 per cent in excess of the calculated quantity. This proportion of excess of the precipitant has been used in all the work. After precipitation, two procedures are available. In either case several hours are allowed for precipitation to become complete. In this laboratory it has always been placed in the ice box over night. Then, if one follows the original method of Windaus,[2] it is filtered on a Gooch filter, washed with alcohol and ether, dried in the air bath, and weighed. Since the compound is known to be slightly

soluble in alcohol, Fraser and Gardner[6] have modified the Windaus technique by washing with ether followed by boiling water, thus avoiding the use of alcohol in washing, and they use either a Gooch filter or tared filter papers in which the tared paper is washed with ether and water parallel with the paper containing the precipitate. This latter method has several advantages over the Windaus technique. In the first place, once the precipitate becomes firmly packed down on a Gooch filter, subsequent filtration and washing is very slow indeed; moreover, by the use of filter papers, several determinations can be carried out at the same time. The avoidance of alcohol washings probably obviates a slight loss in weight, since the precipitate is somewhat soluble in it. On the other hand, the washing with boiling water is probably a much more effective means of removing excess digitonin from the precipitate. Digitonin in a 1 per cent solution quite readily crystallizes out, and will not again go into solution until the mixture is heated up to about 60°. Therefore, if such a crystallizing out of digitonin should occur in a determination which was subsequently washed with cold alcohol and ether, it would almost certainly not dissolve, and the result would of course be too high. The procedure of Fraser and Gardner has, therefore, been followed in all practical determinations made in this laboratory. The weight of the precipitate is multiplied by the calculated factor 0.2431 to obtain the weight of cholesterol present rather than the approximate factor 0.25 recommended by Windaus to cover loss of the compound in washing.

In four analyses the results were as follows:

Cholesterol.		Remarks.
Taken.	Recovered.	
gm.	*gm.*	
0.0200	0.0204	Filtered on tared filter papers and washed with ether and boiling water.
0.0200	0.0205	
0.0200	0.0198	Filtered on a Gooch filter and washed with alcohol and ether.
0.0200	0.0201	

Since, then, accurate results are to be obtained by either of the two procedures as long as pure cholesterol is used to begin with,

[6] Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc., Series B*, 1909, lxxxi, 230.

what is the explanation of the marked discrepancy in analyses of blood? The greatest possibility for error, of course, lies in the preliminary extraction method. A complete extraction cannot be attained by extracting blood directly with an immiscible solvent, because of the formation of emulsions, and also because cholesterol apparently forms some kind of a physical or loose chemical combination with the proteins, from which it cannot be removed by such solvents as ether and chloroform. If the first difficulty is overcome by drying the blood with anhydrous sodium sulfate and grinding, and then extracting with ether in a Soxhlet, still the removal is incomplete.

In the Autenrieth-Funk method the completeness of the extraction depends on the preliminary breaking up of the protein molecules themselves by digesting on a water bath for 2 hours with 25 per cent potassium hydroxide in the proportion of about one part of blood to ten of alkali. This mixture will then give up the cholesterol to either chloroform or ether. Of course in such a process, the esters of cholesterol are saponified, so that it would be useless to extract in this way for the digitonin method, in which the main point is to determine separately the free and combined cholesterol.

If blood is added directly to alcohol, and the precipitate thus obtained extracted with boiling alcohol or by ether, or if blood is run slowly into a mixture of three parts alcohol to one of ether, using three parts of blood to one hundred of solvent, and the whole then brought to a boil on a water bath (Bloor's method) the extraction will be found to be practically complete, as shown by qualitative tests of the protein residue after filtration.

The following experiments will serve to illustrate the foregoing points:

Two 20 cc. portions of dog blood were dried with sodium sulfate and finely ground. These were then extracted in a Soxhlet apparatus for 1 and 5 days respectively, and the cholesterol was determined in the extracts by the digitonin method. Duplicate portions of 2.5 cc. each were analyzed colorimetrically by the method of Autenrieth and Funk.

	per cent
Colorimetric values, A	0.289
B	0.287
Gravimetric values, 1 day	0.125
5 days	0.150

Several specimens of blood were analyzed both colorimetrically using the method of Autenrieth and Funk, and in some instances also that of Bloor, and gravimetrically after precipitating the sample (10 cc.) with alcohol, and extracting the precipitate 24 hours with boiling alcohol in a continuous extraction apparatus, then concentrating the combined alcohol extracts, diluting with water, and extracting with ether.

Following are a few typical analyses:

Source.	Autenrieth-Funk.	Bloor.	Digitonin.
	per cent	*per cent*	*per cent*
Dog	0.185		0.151
"	0.231	0.267	0.169
Horse	0.151	0.186	0.100

The protein residues, after extraction with boiling alcohol, were digested for 2 hours with 100 cc. of 25 per cent potassium hydroxide, cooled, and extracted with 50 cc. of chloroform, the chloroform concentrated to about 5 cc., and to this 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid were added. Only a very faint yellowish green color was obtained, showing practically a complete extraction. The color obtained was about equal to that produced by about 0.0001 gm. of cholesterol in the same volume.

The above figures illustrate the lower results obtained by the digitonin method, even when extraction was probably complete. It did not, however, seem quite fair to make comparisons which would leave room for question on this score. Several experiments, of which the following are types, were carried out.

12 cc. of sheep blood were digested for 2 hours with 100 cc. of 25 per cent potassium hydroxide, and the resulting liquid was extracted once with 250 cc. of chloroform and three successive times with 125 cc., shaking vigorously for 5 minutes each time. (This proportion of chloroform to solution and time of shaking are recommended by Autenrieth and Funk.) The combined extracts were shaken out with two small portions of water to decolorize, and then dried over sodium sulfate. After concentrating, the residue was transferred to a 100 cc. volumetric flask, filled to the mark, and 16.7 cc. (= 2 cc. of blood) were removed for colorimetric analysis. The remainder (= 10 cc. of blood) was evaporated to dryness, taken up in alcohol, and precipitated by digitonin. A second portion of blood was analyzed colorimetrically by Bloor's method.

	per cent
Colorimetric result	0.136
Gravimetric "	0.114
Colorimetric " (Bloor)	0.182

The experiment was repeated with 12 gm. of sheep liver, the alkaline mixture being autoclaved 1 hour at 15 pounds' pressure, instead of being boiled for 2 hours.

	per cent
Colorimetric result	0.409
Gravimetric "	0.374

Dog blood digested as in the case of the sheep blood gave the following figures:

	per cent
Colorimetric result	0.182
Gravimetric "	0.152
Colorimetric " (Bloor)	0.216

Bloor states, in accounting for his higher results, that probably a portion of the cholesterol is destroyed by the long continued boiling with strong alkali in the Autenrieth-Funk method. This is a reasonable assumption, and, if true, would equally well explain the lower gravimetric figures. In order to bring about extraction and saponification without the use of such strong alkali the following experiments were done:

Two samples of dog blood were obtained, and 15 cc. portions were extracted by the method of Bloor, using, for each, 500 cc. of a mixture of three parts alcohol and one part ether, and adding the blood slowly to about 400 cc., bringing to a boil, cooling, and making up to the mark in a 500 cc. volumetric flask. The mixture was filtered by suction and the precipitated protein sucked as dry as possible, but not washed. The filtrate was made up to 500 cc. A 5 cc. portion was removed for colorimetric determination. The remaining 495 cc. were in each case evaporated down to 15 to 20 cc., taken up in water, and thoroughly extracted with four portions of ether. The aqueous residue after ether extraction was evaporated to dryness, 25 per cent potassium hydroxide added, and heated for a half hour on the water bath, then extracted with chloroform. The chloroform extract concentrated to about 5 cc. gave only a trace of yellowish color with acetic anhydride and sulfuric acid; hence extraction was complete. The ether extracts were united, concentrated, and made up to 100 cc. A portion equivalent to 2 cc. of the blood was removed for colorimetric analysis, the remaining ether evaporated off, and the residue (= 13 cc. blood) saponified for 2 hours with 50 cc. 0.5 N alcoholic potash under a return condenser. The greater part of the alcohol was then evaporated off, the residue taken up in water, and thoroughly extracted with ether. The aqueous residue again showed no cholesterol when tested as described above. The ether extracts were concentrated, made to 100 cc., a portion equivalent to 2 cc. of blood was removed for colorimetric analysis, a further portion equivalent to 1 cc. removed and discarded, the remaining ether

extract, equivalent to 10 cc. of blood, evaporated to dryness, and the residue taken up in alcohol and precipitated with digitonin.

Determinations were also made on the blood by the Autenrieth-Funk method.

The results were as follows:

	I.	II.
	per cent	*per cent*
Colorimetric result (Bloor's method, 5 cc. of extract)	0.228	0.293
" " ether extract, unsaponified	0.176	0.233
" " " " saponified	0.174	0.237
Gravimetric "	0.159	0.208
Colorimetric " Autenrieth-Funk method	0.197	0.239

With the exception of the figure obtained by the Autenrieth-Funk analysis of Sample I, which is apparently too high, the figures compare very well with those of the preceding experiments. The striking decrease in the colorimetric value produced only by extracting the concentrated alcohol-ether solution with ether, and thus eliminating some brownish pigment which always is present in the latter, is shown well in both sets of analyses, and there is apparently no further loss after saponification. All of the material, however, which contributes to the green color is evidently not cholesterol, for it cannot be precipitated by digitonin.

There was still the chance that the 2 hours' saponification with alcoholic potash so altered some of the cholesterol that, while it still gave the color reaction it failed to precipitate with digitonin.

To see if this were the case, two samples of 10 cc. each of horse blood were extracted by Bloor's method, and to the alcohol used in one case 0.02 gm. of pure cholesterol was added. These two extracts were saponified and precipitated as above, the resulting amounts of cholesterol obtained being respectively 0.0076 and 0.0274 gm. There was consequently 0.0198 gm. of the cholesterol recovered in spite of the 2 hours' saponification. This made it pretty certain that there was little change in the cholesterol by the alkali, for by mass action more cholesterol should have been changed in the second case than in the first, if a change took place, and a greater loss would have resulted. But to avoid any error on this point, the effect of saponification with 0.5 N alcoholic potash was tried on pure cholesterol. Three portions of 0.0100 gm. of cholesterol in 10 cc. of alcohol were pre-

pared. One portion was immediately precipitated with digitonin. The second was saponified for 1 hour, and the third for 2 hours, then extracted and precipitated. The amounts recovered were respectively 0.00958, 0.00948, and 0.00943 gm., so that there was practically no loss after 2 hours' saponification.

In a number of determinations the ether washings from the digitonin-cholesterol precipitate were extracted once or twice with water to remove the excess digitonin, evaporated to dryness, taken up in 5 cc. of chloroform and acetic anhydride, and sulfuric acid was added. After a short time an unmistakable olive green color always appeared, having, however, a brownish cast. This was equal in intensity perhaps to that which would be produced by 0.002 gm. of cholesterol, and, since the precipitate of cholesterol in the average experiment was usually about 0.015 gm., it amounts to considerably more than 10 per cent of the total color which could have been produced by the material before precipitation. The green color produced by these filtrates faded out rather more quickly than the cholesterol color, and left a yellowish brown solution. In one case, where the residue was treated with glacial acetic acid and a few drops of concentrated sulfuric acid, a slightly reddish color was produced (oxycholesterol reaction?—see below).

DISCUSSION.

It is apparent from the foregoing that the values obtained by the colorimetric method represent some additional substance or substances beside cholesterol itself. That substances closely related to cholesterol exist in the blood normally is shown by the work of Lifschütz.[7] So called "oxycholesterol," a substance which may be prepared artificially from cholesterol by oxidation with potassium permanganate, ferric chloride, or benzoyl peroxide in glacial acetic acid solution, and characterized chemically by the production of a reddish violet color passing into green, with definite spectrum lines upon the addition of sulfuric acid to its acetic acid solution, has been isolated by him from the blood, and a method worked out for its quantitative determination.

[7] Lifschütz, J., *Z. physiol. Chem.*, 1906-07, l, 437; 1907, liii, 140; 1908, lviii, 175.

There is some question as to the properties of these oxidation products, of which there are two or three which can be recognized spectroscopically. Lifschütz[8] states that they will give the Liebermann-Burchard color reaction, and also that they will precipitate with digitonin from alcohol solutions, while Schreiber[9] denies the latter point. Since much of this oxycholesterol work remains as yet unconfirmed it is scarcely desirable to speculate about the exact part which is played by these derivatives in the results obtained in "cholesterol" determinations. Certain it is that they, or some related substances present in the blood, add to the green color of the Liebermann-Burchard test, but fail to precipitate with digitonin. It is quite possible that there may be a partial precipitation of some of this material by the digitonin, and that the gravimetric results themselves are somewhat in excess of the actual amount of true cholesterol present. No attempt has been made to determine whether such a condition exists or not. In one experiment it was shown that there was no wide divergence from the theoretical formula for the precipitate by boiling a sample weighing 0.0111 gm., which had been obtained from dog blood, with 0.5 N alcoholic potash for 2 hours, which gradually dissociates the compound and destroys the digitonin. After a thorough ether extraction of the residue, there could be recognized colorimetrically 0.0026 gm. of cholesterol, while the amount present theoretically was 0.0027 gm. Of course the presence of any oxycholesterol-digitonin compound is not disproved by such an experiment, since the formulas would be closely related, cholesterol being $C_{27}H_{44}O$ and oxycholesterol $C_{27}H_{44}O_2$. For the present, however, it seems warranted to look upon the digitonin determinations of cholesterol in blood as being very nearly correct values when care is taken to insure complete extraction. Values obtained by colorimetric methods should be considered as representing cholesterol plus some more or less closely related substance or substances, very likely of the nature of oxidation products.

[8] Lifschütz, *Münch. med. Woch.*, 1913, lx, 1549, 2346.
[9] Schreiber, E., *Münch. med. Woch.*, 1913, lx, 2001.

CONCLUSIONS.

1. When applied to solutions of pure cholesterol either the colorimetric or the digitonin method of determination is accurate.

2. Ether extraction of dried blood, even when prolonged, is not complete, and recourse must be had to hot alcohol or alcohol and ether.

3. Colorimetric analyses of blood give results too high for true cholesterol, because they include other ether- and chloroform-soluble substances, whereas digitonin determinations are more nearly correct.

THE INFLUENCE OF AUTOLYSIS UPON CHOLESTEROL ESTERS.

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It has been shown[1] that cholesterol is absorbed into the animal body from the intestinal tract by way of the thoracic duct. No matter whether it is fed in the free condition or in the form of esters of the higher fatty acids, it is always found to have been converted to a mixture of about one part free to three or four parts ester by the time it reaches the chyle.

There are probably two quite distinct functions served by these two forms. Nothing can be said of the use of the esters of cholesterol, while in the free condition it is known to act at least *in vivo* as a neutralizing substance to a number of different poisons, such as saponin,[2] tetanolysin,[3] a hemolytic agent of the pneumococcus,[4] and also conceivably, poisons of metabolic origin. It seems desirable then, to study any further interchange between the two forms which may take place in the body.

A review of results reported by many different observers shows clearly that the total cholesterol of the blood serum of practically all species of animals reported upon (exclusive of human serum under pathological conditions) is always made up of both free and combined fractions, 25 to 35 per cent of the total being free. It will be noted that this is the proportion in which the cholesterol appears in the chyle after its absorption. When analyses of the red or white blood cells or of practically any

[1] Mueller, J. H., *J. Biol. Chem.*, 1915, xxii, 1.
[2] Ransom, F., *Deutsch. med. Woch.*, 1901, xxvii, 194.
[3] Noguchi, H., *Univ. Pennsylvania Med. Bull.*, 1902, xv, 327.
[4] Cole, R., *J. Exp. Med.*, 1914, xx, 346.

normal tissue, are made, the results are entirely different. In the red cells,[5] for example, there is probably no combined cholesterol; very little is found in the white cells, and the same is true for most other tissues. The brain contains practically no esters;[6] the kidney,[7] heart,[8] and liver contain varying proportions, from one-tenth to one-half of the total.

There are possibly two explanations for such a condition. Either the body cells are able by means of enzymes to split the esters of the blood plasma, and absorb the free cholesterol, or they absorb the free cholesterol selectively, and the esters are destroyed or altered in some way other than by simple hydrolysis. The first supposition seems the more reasonable, and experiments directed at the recognition of such enzymes have been carried out. It may be stated here that the results have been negative, but for reasons which will appear later it seemed worth while to present the material briefly at this stage.

The attempt has been made to show, by means of autolysis of blood, liver, and mixtures of the two, that there occurred hydrolysis of the esters of cholesterol which normally exist in such materials. This form of experiment and the selection of this material was suggested by two reports in the literature, and it was hoped to make these the basis for more extended work.

Schultz[9] autolyzed human, horse, and dog blood, and also liver and mixtures of blood and liver in the presence of sodium fluoride and thymol or chloroform as preservatives. He was unable to get any evidence of hydrolysis in blood alone, but in horse liver alone and in mixtures of horse, and, to a less extent, of sheep blood and liver, marked hydrolysis took place. Cytronberg,[10] in the same laboratory, reported positive results on horse and dog blood alone, and ascribed Schultz's failure to insufficient familiarity with the digitonin method of analysis. This work has

[5] Wacker, L., and Hueck, W., *Arch. exp. Path. u. Pharm.*, 1913, lxxiv, 416.

[6] Lapworth, A., *J. Path. and Bact.*, 1911, xv, 254.

[7] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.

[8] Hess-Thaysen, T. E., Habilitationsschrift, Copenhagen, 1913; abstracted in *Centr. Biochem. u. Biophysik.*, 1913–14, xvi, 85.

[9] Schultz, J. H., *Biochem. Z.*, 1912, xlii, 255.

[10] Cytronberg, S., *Biochem. Z.*, 1912, xlv, 281.

remained unconfirmed, except for the statement of Gardner and Lander[11] that in an autolyzed cat liver, no cholesterol esters could be found.

There are certain peculiarities about the figures quoted by Schultz and by Cytronberg which may be mentioned briefly. Both workers analyzed equal quantities of blood, usually 200 cc. before and after autolysis. Corper[12] and Thiele[13] have shown that total cholesterol is not decreased on autolysis of tissues. Schultz's figures, however, nearly always show a loss of a third to a half of the total cholesterol after 4 days' autolysis, and in one case of blood and liver, a loss of two-thirds. This loss is not mentioned in his discussion of his results. It is hard to explain so much loss, even by incomplete extraction, but no other explanation is apparent. The figures of Cytronberg do not show this decrease, but his analyses of fresh blood are most peculiar in that they show only about 30 per cent of the total cholesterol to be in the free condition. From analyses of whole blood, together with analyses of plasma, he calculates in two instances that the red cells contain about 60 per cent of their total cholesterol as esters. Most analyses available for reference place the free cholesterol in whole blood at between 60 and 70 per cent of the total. The figures given below for fresh blood illustrate this well. Furthermore, it is now generally recognized that the red cells contain cholesterol esters only in traces, if at all. Apparently, then, the analytical results in both papers are open to criticism.

EXPERIMENTAL.

All blood and tissue used were obtained as fresh as possible, and always used within an hour. Blood was diluted with an equal volume of water before autolysis. In mixtures of liver and blood, the finely ground liver was mixed with an equal weight of blood, and one volume of water was added. For preservatives sodium fluoride, chloroform, tricresol, and toluene were used, in various experiments. It may be said that in some of the experiments, in which liver was autolyzed, there was apparently so

[11] Gardner, J. A., and Lander, P. E., *Biochem. J.*, 1913, vii, 576.
[12] Corper, H. J., *J. Biol. Chem.*, 1912, xi, 44.
[13] Thiele, F. H., *Biochem. J.*, 1913, vii, 275.

much putrefaction, in spite of a reasonable amount of preservative, that positive results would have been questioned, even if obtained. The effort was made in all the work to use the minimum amount of antiseptic effective to prevent putrefaction, in order to avoid enzyme inhibition as far as possible. In most cases extraction has been carried out with boiling alcohol, but in a few cases ether has been used after drying with sodium sulfate. Such results are probably too low, but, even so, marked hydrolysis should have been apparent. The alcoholic extracts are concentrated, diluted with water, and extracted by ether. The ether extract is divided into equal parts, one-half saponified with alcoholic potash for 2 hours, and again extracted. After evaporation of the ether from both fractions, each is taken up in 10 cc. of alcohol and precipitated by digitonin. The weight of the digitonin-cholesteride multiplied by 0.2431 gives in one case the free cholesterol and in the other the total.

The figures in the column headed "ratio" are obtained by dividing the percentage of total cholesterol in the material by the percentage of free.

DISCUSSION.

Slight variations in the results obtained for total cholesterol before and after autolysis are attributable to experimental error in the method. In none of the experiments is there any such evidence of hydrolysis as has been reported by Schultz and Cytronberg. In one experiment quoted by the latter the ratio of free to total cholesterol rose from 36.6 to 91.7 during 4 days' autolysis, and other figures were almost as striking. It can only be concluded from the figures here quoted, that a careful repetition of their work has entirely failed to confirm their results.

It is hoped that, later on, further work by different methods may throw some light on the main question of whether or not the tissues during life can hydrolyze the esters of cholesterol.

Material.	Amount.	Total.	Free.	Ratio.	Remarks.
	cc.	*per cent*	*per cent*	*per cent*	
Ox blood, fresh.....	20	0.120	0.081	67.5	$CHCl_3$ and NaF.
4 days' autolysis	20	0.108	0.084	77.7	
Ox blood, fresh.....	20	0.099	0.050	50.51	$CHCl_3$ and NaF.
4 days' autolysis.....	20	0.094	0.047	50.00	
Sheep blood, fresh....	20	0.051	0.031	61.54	
4 days' autolysis.....	20	0.056	0.032	56.90	$CHCl_3$ and NaF.
4 " "	20	0.057	0.033	57.63	NaF.
Dog blood, fresh.....	20	Lost.	Lost.		
4 days' autolysis.....	20	0.123	0.081	65.66	$CHCl_3$ and NaF.
4 " "	20	0.131	0.082	62.76	NaF.
Fresh.....	20	0.169	0.102	60.11	Same dog bled 3 days later.
Horse blood, fresh....	20	0.100	0.065	65.00	
4 days' autolysis.....	20	0.099	0.060	60.54	$CHCl_3$ and NaF.
	gm.				
Sheep blood and liver, fresh.....	25	0.242	0.125	51.82	Measurement of amount taken only approximate.
4 days' autolysis.....	25	0.300	0.162	53.93	NaF, tricresol, and $CHCl_3$.
Horse blood and liver, equal parts, fresh..	25	0.123	0.107	87.01	
4 days' autolysis.....	25	0.116	0.105	90.65	NaF and $CHCl_3$.
	cc.				
Horse blood citrated, fresh.....	20	0.108	0.087	80.55	Ether extraction. Note increase in total amount extracted after autolysis.
1 month's autolysis...	20	0.132	0.082	62.15	Tricresol.
Horse blood, defibrinated, fresh.....	20	0.114	0.092	80.70	Tricresol.
1 month's autolysis....	20	0.159	0.102	64.77	"
		gm.	*gm.*		
Horse blood and liver, equal parts, fresh..		0.1086	0.1033	95.1	Ether extraction.
4 day's autolysis.....		0.0399	0.0036	90.9	Tricresol and toluene.

STUDIES ON BLOOD SERUM.

I. THE DETERMINATION OF NON-COLLOIDAL NITROGEN.

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INTRODUCTION.

In recent years, knowledge regarding the amount of non-colloidal nitrogen present in the blood has become of more and more importance to the clinician and the biochemist. In connection with our work on the Abderhalden serum reaction, it appeared to us that a quantitative determination of the non-colloidal nitrogen formed by the tryptic activity of the serum would be of more value than a qualitative test on the same by the ninhydrin reaction.

Folin and Denis[1] proposed a method for the determination of the non-protein nitrogen of the blood in which methyl alcohol is used as the precipitant, and the filtrate is subsequently treated with zinc chloride for the removal of some colloidal nitrogenous substances that are not precipitated by the methyl alcohol under the conditions of the experiment. Believing that aluminium hydroxide cream[2] had certain advantages over methyl alcohol as a precipitant for the colloids of the serum, we selected it as our reagent for this purpose.

EXPERIMENTAL.

In this study, we compared the results obtained by the use of the method described by these investigators with those obtained by the use of the aluminium hydroxide cream method. The

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

[2] Marshall, J., and Welker, W. H., *J. Am. Chem. Soc.*, 1913, xxxv, 820. Tracy, G., and Welker, W. H., *J. Biol. Chem.*, 1915, xxii, 55.

latter method was carried out as follows: 5 cc. of serum were accurately measured into a 250 cc. volumetric flask. 75 cc. of aluminium cream were then transferred to the flask and the contents were mixed by shaking, and following this were diluted to the mark with distilled water. A thorough mixing of the contents was followed by filtration. The total nitrogen was determined in 125 cc. of the filtrate by the Kjeldahl method. In all cases, the filtrates from the serum treated with aluminium cream failed to show any protein by Heller's ring test or by the coagulation test. A blank determination was run on the aluminium cream and this result was subtracted from that obtained with the aluminium-treated serum.

The aluminium cream method is simpler in technique than that suggested by Folin and Denis, since only one filtration is required. There is less danger of error through evaporation of the solution. Our results, which are averages of two or more closely agreeing determinations, show the recovery of non-colloidal nitrogen when added to serum. The substances first studied were urea, creatine, glycocoll, and leucine (Table I).

TABLE I.

Method.	Serum added.	Urea.	N.*	Glycocoll.	N.*	Leucine.	N.*	Creatine 1.	N.*	Creatine 2.	N.*
	cc.	*cc.*	*mg.*	*cc.*	*mg.*	*cc.*	*mg.*	*cc.*	*mg.*	*cc.*	*mg.*
Kjeldahl		2.5	14.07	2.5	2.24	2.5	0.70	2.5	1.75	2.5	1.54
Aluminium (Kjeldahl)		5	14.07	5	2.24	5	0.70	5	1.68	5	1.36
" "	5	—	0.98	—	1.12	—	1.12	—	0.91	—	0.84
Alcohol "	5	—	1.12	—	1.26	—	1.26	—	0.98	—	0.98
Aluminium "	5	5	14.95	5	3.29	5	1.75	5	2.24	5	2.02
Alcohol "	5	5	15.18	5	3.42	5	1.89	5	2.17	5	2.10
Aluminium (by difference)		5	13.97	5	2.17	5	0.63	5	1.33	5	1.18
Alcohol " "		5	14.06	5	2.16	5	0.63	5	1.19	5	1.12

* These results, in all cases except where the Kjeldahl method was applied directly, are equivalent to one-half the volume of the samples indicated, since the determinations were carried out on one-half the volume to which the samples were originally made up.

In a subsequent series of experiments, in order to make our conditions approximate those in the Abderhalden test, we used solutions which we have designated Dialysates 1 and 2. These

solutions were obtained by subjecting dog serum to tryptic digestion at 40°C. and dialyzing the resultant mixture in parchment paper against distilled water. The dialysates were free from the more complex protein compounds as evidenced by negative Heller's ring and coagulation tests. Neither of the dialysates showed a precipitate with picric acid, but they gave a faint biuret and a very intense ninhydrin test. These dialysates represented a mixture of protein cleavage products similar to those obtained in the Abderhalden tests. The results are shown in Table II.

TABLE II.

Method.	Serum.	Dialysate.	Nitrogen.*	
			1.	2.
	cc.	*cc.*	*mg.*	*mg.*
Kjeldahl		2.5	0.84	1.33
Aluminium (Kjeldahl)		5	0.74	1.12
" "	5	—	1.33	0.91
Alcohol "	5	—	1.06	0.98
Aluminium "	5	5	2.17	2.10
Alcohol "	5	5	1.82	2.17
Aluminium (by difference)		5	0.84	1.19
Alcohol " "		5	0.76	1.19

* These results, in all cases except where the Kjeldahl method was applied directly, are equivalent to one-half the volume of the samples indicated, since the determinations were carried out on one-half the volume to which the samples were originally made up.

DISCUSSION.

The results show that the nitrogen of urea, glycocoll, and leucine can be recovered to a very large extent, and that creatine nitrogen can be recovered only in part by this method. In the experiments on the solution of the non-colloidal products of the tryptic digestion of the serum proteins there is also a failure to recover all the added nitrogen. The results obtained by the use of this method show that not all the non-colloidal nitrogen is determined. They follow closely, however, those obtained by the use of the Folin and Denis method.

CONCLUSIONS.

Aluminium cream is a satisfactory reagent for the removal of the colloidal nitrogen from blood serum. Its use simplifies the determination of the non-colloidal nitrogen of the serum. The liability to experimental error is less than in the method proposed by Folin and Denis.

THE MECHANISM OF THE SPARING ACTION OF CARBOHYDRATES ON PROTEIN METABOLISM.

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Voit,[1] Rubner,[2] Benedict,[3] Sivén,[4] Landergren,[5] and others have repeatedly demonstrated that ingested carbohydrates exert a sparing effect on the metabolism of body protein. This sparing action is considerably more marked with carbohydrates than with fats. It is possible with the ingestion of large amounts of carbohydrates to reduce the nitrogen output in man from the starvation figures of 8 or 10 gm. to as low as 3 gm. If the carbohydrates are omitted from the diet or even replaced by fat, the nitrogen output again increases to more than double this minimum. Loewi,[6] Lüthje,[7] Lesser,[8] and others further showed that carbohydrates are necessary for the utilization of the split products of protein. Without carbohydrates there is no retention of nitrogen on feeding mixtures of the abiuret split products. In explanation of this, Lüthje thought that an amino sugar was formed. There is, however, no experimental evidence to support this view. According to Landergren, since under physiological conditions fats cannot supply this want, the proteins are broken down to yield the sugar

[1] Voit, C., Hermann's Handb. Physiologie, Leipsic, 1881, 140.

[2] Rubner, M., Die Gesetze des Energieverbrauchs bei der Ernährung, Vienna, 1902.

[3] Benedict, F. G., *Carnegie Institution of Washington, Publication No. 77*, 1907.

[4] Sivén, V. O., *Skand. Arch. Physiol.*, 1900, x, 91; 1901, xi, 308.

[5] Landergren, E., *Skand. Arch. Physiol.*, 1903, xiv, 112.

[6] Loewi, O., *Arch. exp. Path. u. Pharm.*, 1902, xlviii, 303.

[7] Lüthje, H., *Arch. ges. Physiol.*, 1906, cxiii, 547.

[8] Lesser, E. J., *Z. Biol.*, 1904, xlv, 497.

which is lacking. This results in an increase in the nitrogen output. Cathcart[9] suggested that carbohydrate is essential to protein synthesis, and this view is held also by Janney.[10]

Sugar, except when stored as glycogen, is dissociated in a definite way in the body into simpler molecules. An idea of the relationship of glucose to the dissociation products may be gathered from the following scheme:

				Oxidation.		
$C_6H_{12}O_6$	$\rightarrow$	CH_3COCHO	$\rightarrow$	$CH_3CHOHCOOH$	$\rightarrow$	$CH_3COCOOH$
Glucose.		Methyl glyoxal.		Lactic acid.		Pyruvic acid.

Of special interest in this connection is the fact demonstrated by Otto Neubauer that keto and hydroxy acids identical with those arising in the course of sugar catabolism may result from the dissociation of proteins in the body. For example, alanine is deaminized to form pyruvic acid, which by reduction gives rise to lactic acid according to the following scheme:

$CH_3CH(NH_2)COOH$	$\rightarrow$	$CH_3COCOOH$	$\rightarrow$	$CH_3CH(OH)COOH$
Alanine.		Pyruvic acid.		Lactic acid.

It has been shown by a number of workers that the above reactions are reversible. For example, lactic acid and pyruvic acid may give rise to glucose in a phlorhizinized animal. The increased glycosuria in phlorhizinized animals following ingestion of proteins is a further evidence of the reversal of this reaction. That the second of these reactions is reversible, namely, the synthesis of hydroxy and keto acids into amino-acids, has been demonstrated by Knoop[11] as well as by Embden[12] and others. They have shown that on perfusing the isolated liver with lactic or pyruvic acid, nitrogen can be added on and alanine formed. If this process of retaining nitrogen by dissociation products of sugar to form new amino-acids, and hence proteins, occurs on a large scale in the body, it will explain why ingestion of carbohydrates

[9] Cathcart, E. P., The Physiology of Protein Metabolism, London, 1912, 121.

[10] Janney, N. W., *J. Biol. Chem.*, 1916, xxiv, p. xxx.

[11] Knoop, F., *Z. physiol. Chem.*, 1910, lxvii, 489.

[12] Embden, G., and Schmitz, E., *Biochem. Z.*, 1910, xxix, 423.

spares body protein. To test this point, the sparing effect on the nitrogen output of ingesting lactic and pyruvic acids as compared with the sparing action of equivalent amounts of undissociated carbohydrates was undertaken in these experiments:

EXPERIMENTAL.

Large healthy dogs were used for the experiments. They were allowed to fast for 5 or 6 days. When the nitrogen output became constant, lactic acid, pyruvic acid, or cane sugar was given by stomach tube. The lactic acid was partly neutralized by calcium hydroxide; pyruvic acid was neutralized with sodium hydroxide. For comparative purposes the sparing action of cane sugar was first determined for each dog. The urine was collected every 24 hours by catheterization. During fasting the dogs received daily a liter of water, and the carbohydrates and acids were administered in an equivalent dilution, so that the daily quantity of urine was kept constant. The results of the experiments are tabulated in Tables I to III.

TABLE I.

Experiment 1.—A healthy bitch weighing 19.5 kg. Cane sugar was given on the 7th day; lactic acid partly neutralized with calcium hydroxide was given by stomach tube on the 10th day.

Day.	Food.	N in the urine.	Remarks.
		gm.	
5	Fasting	2.86	
6	"	2.92	
7	95 gm. cane sugar	1.66	
8	Fasting	2.56	
9	"	2.84	
10	100 gm. lactic acid	1.82	3.32 gm. of *l*-lactic acid were recovered in the urine.
11	Fasting	2.48	
12	"	3.03	

TABLE II.

The Effect of Lactic Acid on the N Output of the Dog.

Experiment 2.—Dog weighing 18.6 kg. In this experiment doses of cane sugar and lactic acid were given over 3 days in amounts of 80 and 80 gm. respectively.

Day.	Food.	N in the urine.	Remarks.
		gm.	
5	Fasting.....	3.35	
6	"	3.49	
7	76 gm. cane sugar...	2.25	
8	76 " " " ...	1.74	
9	76 " " " ...	1.27	
10	Fasting.....	1.44	
11	"	2.22	
12	"	3.19	
13	"	3.40	
14	80 gm. lactic acid...	2.68	Traces of lactic acid found in the urine.
15	80 " " " ...	2.19	" " " " " " " "
16	80 " " " ...	1.65	" " " " " " " "
17	Fasting.....	2.03	
18	"	2.55	

TABLE III.

Experiment 3.—Same dog as in Experiment 2. Pyruvic acid neutralized with sodium hydroxide was given in doses of 80 gm. per day for 3 days. The pyruvic acid was prepared in the laboratory by the distillation of tartaric acid and potassium hydrogen sulfate. It was freshly distilled before use.

Day.	Food.	N in the urine.	Remarks.
		gm.	
6	Fasting.....	3.52	
7	"	3.41	
8	80 gm. pyruvic acid.	2.88	
9	80 " " " .	2.44	
10	80 " " " .	2.18	Traces of *l*-lactic acid were detected in the urine.
11	Fasting.....	2.56	
12	"	2.85	
13	"	3.36	

Following the ingestion of cane sugar there was the usual reduction of the nitrogen output. In these experiments this reduction was down to approximately 53 per cent of the fasting nitrogen when 100 gm. of carbohydrates were given on 1 day only; following the administration of 76 gm. a day for 3 consecutive days, the nitrogen output was reduced to about 37 per cent of the fasting nitrogen. These figures serve as a basis of comparison for the sparing action of equivalent amounts of lactic and pyruvic acids. In Experiment 1 where 100 gm. of lactic acid were given, the nitrogen output was reduced from 2.84 to 1.82 gm., or 64 per cent of the fasting nitrogen. 3.32 gm. of *l*-lactic acid were recovered from the urine. It is apparent that not quite all of the lactic acid had been utilized, but the reduction in the nitrogen output to 64 per cent as compared with 53 per cent for an equivalent amount of cane sugar indicates that lactic acid spares protein to practically the same extent as do carbohydrates. In Experiment 2 lactic acid was given in amounts of 80 gm. on 3 consecutive days. The daily nitrogen output was reduced from 3.4 gm. to 1.65 gm. on the 3rd day, or 48 per cent of the fasting nitrogen as compared with a reduction to 37 per cent following equivalent amounts of cane sugar. There were traces of lactic acid found in the urine, so that it is likely that had all the lactic acid been utilized reduction in nitrogen output would have been fully as great as that following carbohydrates. In Experiment 3 the effect of ingesting pyruvic acid was studied. 80 gm. doses per day were given for 3 days. The nitrogen output fell from 3.41 to 2.18 gm. on the 3rd day, or 64 per cent as compared with 37 per cent following carbohydrates, and 48 per cent following lactic acid. There were no toxic symptoms apparent following administration of these amounts of pyruvic acid in the form of the sodium salt. A trace of *l*-lactic acid was detected in the urine on the 3rd day following the administration.

DISCUSSION OF RESULTS.

Lactic acid exerts practically the same sparing action on protein metabolism as carbohydrates. Following pyruvic acid the sparing action is very distinct but less marked than that following lactic acid and carbohydrates. The dissociation of glucose in

the body is by way of lactic acid as one of the chief intermediate steps. Lactic acid can be oxidized in the body to pyruvic acid. As pointed out in the introductory remarks, it has been shown that lactic acid as well as pyruvic acid can add on ammonia nitrogen to form alanine. When this process is operative, nitrogen arising from catabolism of body proteins instead of being excreted is utilized to synthesize new protein. It is true that the above reactions are reversible, as has been shown in phlorhizinized animals; but the normal catabolism of glucose is in the direction indicated, giving rise to simpler molecules, which in turn may be in part combined with nitrogen to synthesize protein. That this fixing of catabolized nitrogen by the dissociation products of glucose to form new proteins is the true mechanism of the sparing effect of feeding carbohydrates on the nitrogen output gains further support in the experiments here reported.

THE DISTRIBUTION OF THE LIPOIDS ("FAT") IN HUMAN BLOOD.

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Those constituents of the blood which are usually included in the term "blood fat" are (1) the true fats—glycerides of the fatty acids; (2) the phosphatides—lecithin, cephalin, etc.—ordinarily termed "lecithin," and (3) cholesterol with its fatty acid esters. At first these substances were grouped together because of their similar solubilities in the fat solvents, but evidence has been accumulating (see below) which makes it probable that the grouping is a natural one based on their common connection with fat metabolism. For this reason it is increasingly desirable to have a general term which shall include all these substances and also others which are concerned in fat metabolism. Three terms have been used for this purpose. The term "*lipin*" suggested by Gies,[1] although good, has not found general acceptance possibly because the ending *-in* is still associated in most minds with basic properties. Leathes[2] uses the term "*fats*" in the general sense to include all substances connected with fat metabolism. This term has the advantage of quite general application in this sense and would be useful if it were not for the fact that the best known member of the group is also universally known by that name. The third term, "*lipoid*," first used by Overton, has two meanings in the literature—the original meaning given it by Overton including essentially all substances having the solubilities of the fats, and a later restricted meaning including only such substances as "lecithin," cholesterol, etc. At present it is coming into general use in France[3] and to a certain extent in Germany[4] as a general term to include all substances connected with fat metabolism. On the whole, it seems to be the

best of the three terms for the purpose. Its root meaning is broad enough to allow its use in this sense and there is an advantage in the use of a familiar term rather than a new one. It will therefore be used in the present work in the above sense to include all substances connected with fat metabolism; *i.e.*, the fatty acids, their naturally occurring compounds, and also substances like cholesterol which occur naturally in combination with the fatty acids.

As might be expected from the importance of fat in general metabolism a considerable volume of literature has accumulated on the lipoids of the blood, but the published results, although in general agreement, vary greatly in the quantitative details. The reasons for the variations in reported values are, first, the widely different and frequently inadequate methods employed; second, the failure in many instances to take into account the nutritional condition of the subject at the time the blood was taken. Practically the only time when the blood is free from the influence of ingested or mobilized fat and when therefore the fat values are constant enough to be used as a basis for comparison, is the post-absorptive condition—8 to 16 hours after the last meal. A third reason is the probable presence in the blood of enzymes[5, 56] which may alter or destroy the lipoids if the blood is allowed to stand for any considerable length of time after being drawn. For these reasons only a general review of the subject is thought to be of value at the present time.

Fat, Glycerides of the Fatty Acids, Neutral Fat.—Very little is known regarding the amount of fat in the blood at any time. The methods employed for the determination of lipoids in blood are mainly saponification methods giving values only for total fatty acids, and when suitable methods have been employed the analyses are rarely complete so that the amount of fat could not be determined. Letsche's[6] analyses lead him to the conclusion that the amount of fat present in normal fasting blood is small and may be entirely absent. Klemperer[7] comes to a similar conclusion regarding the blood in certain cases of diabetes.

During fat absorption the fat of the blood is increased as a result of the inflow of chyle fat which consists almost entirely of glycerides. Müller[22] finds the fats increased in nephritis, and Klemperer[7] in the lipemia of diabetes.

Total Fatty Acids.—More information is available regarding the total fatty acids of the blood, since most of the methods for determining blood "fat" are saponification methods and involve the separation of the fat as fatty acids; but the values reported for both normal and pathological conditions vary extremely among themselves for the reasons mentioned above.

Very little is known regarding changes in normal human beings in the total fatty acids of the blood other than in the well known alimentary lipemia. In normal animals the total fatty acids have been shown to increase as the result of fasting,[8–12] narcotics,[10, 12, 13] and exercise.[14] The increases in fasting and narcosis are not constant but seem to depend on the nutritional condition of the animal. Animals whose blood lipoid level is ordinarily not affected by fasting or narcosis show marked increases in both conditions after a period of stuffing with fat food. Apparently in these animals some of the fat is so loosely stored that it is thrown into the blood as the result of a stimulus which does not affect the animal in his ordinary condition. Rosenfeld[72] many years ago observed that the fat mobilization ordinarily produced by phosphorus poisoning in dogs does not take place in starving animals.

Fat injected into the circulation in suspension disappears from the blood at a rate which appears to depend on the fineness of the suspension. Injected as a colloidal suspension it disappears in about ½ hour,[15] as casein emulsion (particles 2 to 5 μ in diameter) in less than 5 minutes,[12] as egg yolk, in 6 or 7 hours,[12] as homogenized fat (consisting of particles 0.3 to 1 μ) in about 7 hours.[16]

In pathological conditions in human beings considerable work has been done, particularly in diabetes mellitus. Diabetes is the only common disease in which the blood lipoids are excessively increased and consequently it was the first disease in which attention was directed to these constituents. The milky serum from the blood in many cases of diabetic coma, from which frequently a "cream" arose on standing, suggested fat at once to the observant at the time when blood letting was a common practice, and the earliest information on blood lipoids dates from that time.[17] Our present knowledge regarding the total fatty acids of the blood in diabetes may be summed up as follows.

In diabetics free from acidosis they are not much, if any, higher than in the normal. Acidosis is generally accompanied by increased total fatty acids in the blood but not by any means invariably.[7, 17–21] In coma the values may be very high; 26 per cent is reported by Klemperer,[21] and 15 to 20 per cent appears to be fairly common. On the contrary, lipemia is frequently absent in coma.[21] It seems probable that while the coma and the lipemia may be the result of the same set of conditions the lipemia may develop so gradually that coma may ensue before the lipemia has reached notable proportions. It is questionable whether the increased fat is due to cellular breakdown, as is frequently stated (because of the increase of "lecithin" and other cellular constituents). It seems more reasonable to suppose that the increase represents the accumulation of mobilized or ingested lipoids which the organism is no longer able to utilize.

The "total fatty acids" of the blood have been found to be increased in nephritis,[18, 22] in pneumonia,[18] in pregnancy,[23] and in experimental anemia in animals.[24]

Phosphatides ("Lecithin")—Lecithin, Cephalin, Etc.—The "lecithin" of the blood has been reported to increase in normal animals during the absorption of lecithin[25] and of fat.[26, 27] In pathological conditions in human beings increases in blood "lecithin" have been reported in diabetic lipemia,[21, 28, 29] although here as in the case of the fat the increases do not appear to be constant or characteristic since other cases have been reported,[30, 31] even with coma, where the changes were slight. Other pathological conditions in which abnormal values for "lecithin" have been reported are: nephritis, both high[22, 32] and low values;[28, 32] leukemia, high values[32] (in corpuscles only—plasma normal); syphilis, high values (serum);[28, 33] in cachexia (carcinoma) low values.[34] In experimental conditions in animals increases in blood "lecithin" have been reported in the anemia produced by continued bleeding[24] and in depancreatized dogs.[35]

The divergence in the results of lecithin determinations in blood, particularly the low values, may be due to one or both of two causes: (1) destruction of lecithin in the blood by standing, due to the action of an esterase in the corpuscles;[5] or (2) destruction during extraction. Many of the blood samples reported on were obtained post mortem and therefore after standing several

hours; also the methods used for extraction in most cases would undoubtedly result in a partial hydrolysis.

Cholesterol.—Cholesterol is a relatively well defined and stable chemical substance, is readily extracted from the blood and purified, and is readily acted upon by a number of reagents producing precipitates or colors which may be used for its quantitative determination. For these reasons it has been the subject of a good deal of investigation in recent years. Also by reason of its stability the values reported for cholesterol in blood are much more comparable than those for "lecithin," although here again the lower values may be regarded as doubtful, either because of incomplete extraction or because of the action of the strong alkali used for hydrolysis preparatory to extraction in most of the methods, since cholesterol is not entirely stable in the presence of strong alkalies.[36] Cholesterol occurs in the blood in two forms; "free" in the corpuscles and to some extent in the plasma, and as cholesterol esters in the plasma alone. The relation of free to bound cholesterol in the plasma is variously given.[34] One part free to two parts bound may perhaps be taken as a fair average of the values reported. Cholesterol is absorbed from the intestine[37] when fed in solution in oil or in natural solution as in egg yolk, and causes an increase in the cholesterol of the blood.[38, 39] Similar increases in the cholesterol of the blood after feeding fat alone have been reported[26, 40, 41] but these increases have not been confirmed in recent work.[27] Cholesterol has been reported to be increased in narcosis[13, 42] and in alcoholism,[43] also in pregnancy.[23] In pathological conditions cholesterol is increased in apparently all cases of jaundice,[44] presumably because of stoppage of one of its normal paths of excretion. In diabetes there is generally an increase, although as in the case of fat and lecithin the increases are not constant.[45] Increases have also been reported in nephritis[22, 45–47] and lues.[34] Decreases have been noted in cachexia of various origins.[29, 46, 48]

All of these lipoids are probably synthesized by the animal organism. The formation of fat from carbohydrate and probably also from protein is too well known to need discussion here. That "lecithin" is synthesized by the animal organism has been shown by McCollum[49] with rats, by Fingerling[50] and McCollum[51] in feeding experiments with laying ducks and hens, and by

Bloor[27] in fat absorption experiments. As regards cholesterol, Gardner and Lander[52] have found that cholesterol is synthesized in the body of growing chicks, but not readily. Dezani[53] has claimed that it is readily synthesized in the animal body.

The functions of "lecithin" and cholesterol in the organism are little understood, although there can be no question regarding their importance since they are constituents of all living cells and probably constitute most of the "built in" or invisible fat of the tissues. Recent investigations[27] have shown that lecithin probably takes an active part in fat metabolism as the first stage through which the fats pass in their utilization by the organism.

As regards the function of cholesterol there is some evidence that it may serve as food. Mendel and Leavenworth[54] have shown that the cholesterol content of the developing hen's egg diminishes during development. Moreover, there appears to be a considerable cholesterol metabolism in the animal organism, since it or its products are always present in the feces in considerable quantities although it is quite readily absorbed from the intestine.[37] Cholesterol probably has also some function in fat metabolism because of the presence in the blood plasma and in most cells of cholesterol esters whose relation to the cholesterol—in the blood plasma, at least—is claimed to be constant;[38] also because in pathological conditions it increases or decreases along with the other lipoids, preserving a fairly constant relationship with them as has been indicated by the work of Fischer,[17] Müller,[22] Klemperer and Umber,[21] and as shown by the work below. Cholesterol is believed by Lifschütz[55] to be the source of the bile acids.

The presence of a cholesterase in blood corpuscles has been reported by Cytronberg.[56]

In all the work on the blood lipoids noted above there have been very few attempts to make complete analyses of these constituents and still fewer to correlate the results in an effort to determine the distribution of the lipoids between plasma and corpuscles or to discover relationships such as would be expected to occur among substances concerned with a common phase of metabolism. One effort in this direction is the work of Mayer and Schaeffer[57] in the elaboration of their theory of cellular constants. They found that in the red blood cells there is a constant

relationship between the fatty acids and the lipoid phosphorus on the one hand and between the cholesterol and the fatty acids on the other. A similar constancy of relationship between fatty acids and cholesterol in the blood has been reported by Terroine[58] who found besides that the relation remained constant during fat absorption.[41] A fairly constant relationship between total fatty acids and "lecithin" during fat absorption was noted by Bloor.[27]

Having, then, available suitable methods for the approximately complete analysis of the lipoid constituents of the blood, it seemed worth while to undertake in human beings a study of the distribution of the lipoids in both normal and pathological conditions in the effort to discover the normal values and relationships and how these are affected by various diseases, the whole to serve as a basis for future more detailed study of such conditions as show marked abnormalities.

The blood of normal men examined was that of students in this school; that of normal women, of students in the nurses' training course of Simmons College. The pathological blood samples were obtained mainly from patients in the medical wards of the Peter Bent Brigham Hospital. For the work of obtaining most of these samples I am indebted to Dr. D. J. MacPherson of the Peter Bent Brigham Hospital, for those of normal men to Dr. O. F. Rogers formerly of this school. To all of these persons and to the authorities of the hospital I acknowledge my indebtedness and express my thanks for their help in making this work possible.

Having in mind possible changes produced in the blood on standing, the samples were worked up as soon as possible, generally within 2 hours after being drawn from the vein. In the few cases where that was not possible the blood was kept on ice until used. All samples were obtained in the post-absorptive condition, before breakfast. Analyses both of whole blood and plasma were made and from these and the percentage of corpuscles, the lipoid values of the corpuscles were calculated. From the values so obtained the relations between the constituents in whole blood, plasma, and corpuscles were determined.

The routine procedure in the examination of the blood was much the same as that reported in earlier papers[59] and therefore need not be given in detail here. About 10 cc. of blood were drawn from one of the veins of the arm, and run into a gradu-

ated centrifuge tube containing two drops of saturated sodium citrate solution. After thoroughly mixing, a 3 cc. sample was measured out for "whole blood" and run into alcohol-ether as usual. The remainder was centrifuged at 3,800 R.P.M. for 10 minutes, the volume of corpuscles noted, and 3 cc. of the plasma were measured out into alcohol-ether as before.

For the determination of "total fat"[59] 10 cc. of the alcohol-ether extract were adequate in most instances but for the blood of anemia patients 15 cc. were necessary. The method used for the determination of cholesterol has recently been described.[60] 10 cc. of the extract were required for its determination in normal blood and quantities varying from 3 cc. (in severe diabetes) to 15 cc. (in anemia) in pathological blood. The method used for the determination of "lecithin" has also been described recently.[61, 27] In the present work the strychnine molybdate precipitation[62] was used throughout. 15 cc. of the blood extract were required for normal blood and from 10 (severe diabetes) to 25 cc. (anemia) in the pathological samples. The results of the analyses are given in the tables.

TABLE I.

Lipoids of Normal Blood, Gm. per 100 Cc.

	Corpuscles.	Total fatty acids.			Lecithin.			Cholesterol.			Fat.		Total fatty acids / Lecithin			Lecithin / Cholesterol			Total ether-soluble.
		Whole.	Plasma.	Corpuscles.	Whole.	Plasma.	Corpuscles.	Whole.	Plasma.	Corpuscles.	Plasma.	Corpuscles.	Whole.	Plasma.	Corpuscles.	Whole.	Plasma.	Corpuscles.	Plasma.
Men.	*per cent*																		
C. W. B.*		0.29	0.30	0.28	0.29	0.21	0.40	0.21	0.21	0.21	0.05	0.00	1.00	1.43	0.70	1.38	1.00	1.90	0.57
D. E. C.		0.34	0.34	0.34	0.30	0.24	0.38	0.19	0.19	0.19	0.08	0.07	1.13	1.41	0.89	1.58	1.26	2.00	0.60
R. D. C.		0.41	0.43	0.40	0.30	0.26	0.35	0.25	0.31	0.18	0.10	0.15	1.36	1.66	1.14	1.20	0.84	1.94	0.82
E. M. D.		0.34	0.37	0.30	0.31	0.22	0.42	0.20	0.20	0.20	0.12	0.00	1.10	1.68	0.71	1.55	1.10	2.10	0.64
M. F. S.		0.43	0.41	0.45	0.33	0.24	0.44	0.24	0.28	0.19	0.10	0.14	1.30	1.71	1.02	1.37	0.86	2.31	0.76
J. R. L.		0.41	0.40	0.42	0.31	0.21	0.44	0.20	0.21	0.19	0.14	0.11	1.32	1.90	0.95	1.55	1.00	2.32	0.67
R. MacL.		0.37	0.37	0.37	0.29	0.20	0.41	0.23	0.26	0.19	0.10	0.08	1.27	1.85	0.90	1.26	0.80	2.16	0.69
M. M.		0.38	0.37	0.39	0.31	0.20	0.44	0.22	0.21	0.23	0.12	0.08	1.23	1.85	0.90	1.41	0.95	1.91	0.64
C. D. M.		0.37	0.42	0.31	0.31	0.25	0.40	0.21	0.23	0.19	0.13	0.03	1.20	1.68	0.77	1.48	1.09	2.10	0.72
E. P. S.		0.30	0.31	0.30	0.31	0.24	0.40	0.19	0.20	0.18	0.04	0.02	0.97	1.30	0.75	1.63	1.20	2.20	0.58
F. S. T.		0.41	0.42	0.40	0.29	0.22	0.38	0.20	0.21	0.19	0.16	0.13	1.41	1.90	1.05	1.45	1.05	2.00	0.70
H. J. W.		0.33	0.35	0.31	0.30	0.19	0.42	0.19	0.21	0.18	0.12	0.02	1.10	1.84	0.70	1.57	0.90	2.30	0.62
B. P. H.		—	0.37	—	0.29	0.22	0.38	0.20	0.21	0.19	0.11	—	—	1.70	—	1.45	1.05	2.00	0.65
H. M. E.		—	0.41	—	—	0.23	—	—	0.21	—	0.14	—	—	1.80	—	—	1.10	—	0.69
Women.																			
Miss R.	38	0.39	0.44	0.30	0.31	0.22	0.46	0.24	0.26	0.22	0.16	0.00	1.26	2.00	0.65	1.30	0.85	2.10	0.77
" T.	39	0.34	0.39	0.27	0.28	0.21	0.39	0.22	0.22	0.22	0.13	0.00	1.21	1.86	0.69	1.28	0.95	1.80	0.67
" K.	40	0.35	0.41	0.27	0.29	0.19	0.43	0.23	0.25	0.20	0.15	0.00	1.20	2.10	0.63	1.26	0.76	2.15	0.71
" D.	39	0.37	0.39	0.34	0.29	0.19	0.44	0.22	0.25	0.18	0.12	0.03	1.27	2.00	0.77	1.32	0.76	2.44	0.69
" F.	38	0.40	0.45	0.32	0.29	0.18	0.48	0.24	0.24	0.24	0.20	0.00	1.40	2.50	0.67	1.21	0.75	2.00	0.74
" Alb.	35	0.38	0.40	0.32	0.28	0.17	0.48	0.21	0.22	0.19	0.17	0.00	1.36	2.35	0.67	1.33	0.77	2.52	0.67

TABLE 1—*Concluded.*

	Corpuscles.	Total fatty acids.			Lecithin.			Cholesterol.			Fat.		Total fatty acids / Lecithin			Lecithin / Cholesterol			Total ether-soluble.
		Whole.	Plasma.	Corpuscles.	Whole.	Plasma.	Corpuscles.	Whole.	Plasma.	Corpuscles.	Plasma.	Corpuscles.	Whole.	Plasma.	Corpuscles.	Whole.	Plasma.	Corpuscles.	Plasma.
	per cent																		
Mrs. M.	37	0.42	0.47	0.33	0.29	0.21	0.43	0.22	0.24	0.20	0.20	0.03	1.45	2.23	0.77	1.32	0.87	2.15	0.77
Miss Rl.**	37	0.33	0.35	0.30	0.26	0.15	0.44	0.16	0.15	0.17	0.18	0.00	1.27	2.33	0.68	1.62	1.00	2.60	0.54
" A.**	39	0.35	0.38	0.30	0.25	0.14	0.42	0.16	0.15	0.18	0.21	0.00	1.40	2.70	0.71	1.56	0.93	2.33	0.57
Averages.																			
Men		0.36	0.38	0.36	0.30	0.22	0.40	0.21	0.22	0.19	0.11	0.07	1.20	1.68	0.89	1.44	0.96	2.08	0.67
Variations High		0.41	0.43	0.45	0.33	0.26	0.44	0.25	0.31	0.23	0.16	0.15	1.41	1.90	1.14	1.63	1.26	2.32	0.82
Variations Low		0.29	0.30	0.28	0.29	0.20	0.35	0.19	0.19	0.17	0.04	0.00	0.97	1.41	0.70	1.20	0.84	1.90	0.57
Women		0.36	0.40	0.29	0.29	0.19	0.44	0.23	0.24	0.21	0.16	0.01	1.31	2.15	0.69	1.29	0.82	2.14	0.72
Variations High		0.42	0.47	0.34	0.31	0.22	0.48	0.24	0.26	0.24	0.20	0.03	1.45	2.70	0.77	1.33	0.95	2.60	0.77
Variations Low		0.32	0.35	0.27	0.28	0.17	0.39	0.21	0.21	0.19	0.12	0.00	1.20	1.86	0.63	1.21	0.75	1.80	0.57

The value "total fatty acids" is obtained by subtracting the value for cholesterol from that of "total fat" as determined. The value "lecithin" is obtained by multiplying the value obtained for phosphoric acid (lipoid) by 8. "Fat" in the plasma is obtained by subtracting the fatty acids combined as "lecithin" (0.70 × the lecithin value) together with those combined as cholesterol esters (0.48 × the cholesterol value) from the "total fatty acids" and multiplying the result by 1.05; in the corpuscles, by subtracting the fatty acids combined as "lecithin" from "total fatty acids" and multiplying by 1.05 as before. These calculations are made on the assumptions (1) that the lecithin is oleo-stearyl-lecithin; (2) that two-thirds of the cholesterol of the plasma is combined with fatty acid (oleic acid); and (3) that in the corpuscles all the cholesterol is free—assumptions which are believed to be sufficiently near the truth for the purposes of this work. The values given for corpuscles are calculated from those of the whole blood and plasma, taking into account the percentage of corpuscles. The value "total ether-soluble" (plasma) is obtained by adding together the values for "lecithin," cholesterol (including esters), and "fat." The values of the various constituents in plasma and whole blood are believed to be within 5 per cent of the true value; in the corpuscles, since they are calculated values, the variations are greater. Ratios between total fatty acids and lecithin, $\frac{\text{total fatty acids}}{\text{lecithin}}$, and between lecithin and cholesterol, $\frac{\text{lecithin}}{\text{cholesterol}}$, are included in the table, the first because of the relationship observed between "total fatty acids" and "lecithin" during fat absorption,[27] leading to the belief that lecithin is a stage in fat metabolism through which the fats must pass, and the second because of the antagonistic behavior of these two substances observed in several biological processes.[63–65]

* In the case of the normal men where the corpuscle percentage was not determined, 43 per cent (the value given by Keith, Rowntree, and Geraghty[66] as the average for normal men) was used in the calculations. This average value for normal student blood has since been found correct in determinations on other students' blood.

** The lipoids of these two samples were determined last and therefore after this blood had stood for a longer time than the others. Since the values for all the lipoids are lower in these samples it seemed probable that these low values may have been due to enzyme action and they are therefore not included in the averages.

TABLE II.

Lipoids of Pathological Blood, Gm. per 100 Cc.

	Corpuscles.	Total fatty acids.			Lecithin.			Cholesterol.			Fat.		Total fatty acids / Lecithin			Lecithin / Cholesterol			Total ether-soluble.
		Whole.	Plasma.	Corpuscles.	Whole.	Plasma.	Corpuscles.	Whole.	Plasma.	Corpuscles.	Plasma.	Corpuscles.	Whole.	Plasma.	Corpuscles.	Whole.	Plasma.	Corpuscles.	Plasma.
Women.	*per cent*																		
1. Mitral stenosis	44	0.50	0.46	0.55	0.30	0.18	0.45	0.23	0.27	0.18	0.20	0.23	1.67	2.55	1.22	1.30	0.67	2.50	0.78
17. Diabetes	38	0.35	0.35	0.35	0.25	0.15	0.44	0.16	0.16	0.16	0.17	0.04	1.40	2.30	0.80	1.50	0.90	2.70	0.56
47. "	41	0.34	0.36	0.31	0.32	0.17	0.53	0.25	0.27	0.22	0.21	0.09	1.06	2.10	0.60	0.66	1.00	2.10	0.69
2. "	46	0.52	0.57	0.46	0.37	0.30	0.46	0.30	0.38	0.21	0.18	0.13	1.40	1.90	1.00	1.20	0.79	2.20	1.05
70. "	47	0.56	0.70	0.40	0.49	0.48	0.50	0.54	0.65	0.41	0.04	0.05	1.14	1.50	0.87	1.22	0.74	1.20	1.50
3. Debility	44	0.47	0.48	0.46	0.32	0.17	0.51	0.19	0.19	0.19	0.26	0.10	1.47	2.82	0.90	1.70	0.90	2.70	0.71
4. Carcinoma, anemia	23	0.51	0.58	0.28	0.22	0.23	0.19	0.28	0.25	0.38	0.30	0.15	2.32	2.52	1.47	0.79	0.92	0.50	0.90
12. "	36	0.30	0.33	0.27	0.30	0.24	0.40	0.24	0.19	0.33	0.07	0.00	1.00	1.38	0.68	1.25	1.26	1.21	0.59
6. "	34	0.40	0.44	0.32	0.20	0.17	0.26	0.21	0.22	0.19	0.21	0.14	2.00	2.60	1.23	0.95	0.78	1.37	0.71
7. Myocarditis	45	0.41	0.35	0.46	0.29	0.21	0.37	0.26	0.25	0.27	0.08	0.20	1.41	1.67	1.24	1.11	0.84	1.37	0.66
8. Malnutrition	33	0.39	0.26	0.65	0.28	0.20	0.44	0.22	0.25	0.16	0.00	0.34	1.40	1.30	1.48	1.27	0.80	2.80	0.57
9. Nephritis	37	0.53	0.53	0.53	0.33	0.22	0.52	0.24	0.25	0.22	0.26	0.17	1.60	2.41	0.98	1.38	0.88	2.36	0.85
10. Syphilis, alcoholic	34	0.38	0.36	0.42	0.22	0.19	0.28	0.18	0.14	0.26	0.15	0.22	1.73	1.90	1.50	1.22	1.36	1.61	0.55
11. Goiter	43	0.40	0.45	0.33	0.28	0.23	0.35	0.19	0.20	0.18	0.15	0.09	1.42	2.00	0.94	1.47	1.15	1.94	0.68
5. Asthma	33	0.52	0.46	0.64	0.26	0.14	0.49	0.20	0.17	0.26	0.28	0.34	2.00	3.30	1.31	1.30	0.82	1.90	0.67
15. Anemia	12	0.43	0.44	0.36	0.14	0.12	0.29	0.12	0.11	0.19	0.30	0.15	3.00	3.67	1.20	1.20	1.10	1.50	0.59
16. Pernicious anemia	17	0.41	0.44	0.26	0.21	0.18	0.36	0.19	0.18	0.23	0.22	0.00	1.95	2.44	0.72	1.10	1.00	1.13	0.67
21. " "	25	0.44	0.41	0.53	0.24	0.15	0.51	0.21	0.21	0.21	0.16	0.17	1.83	2.73	1.04	1.14	0.70	2.43	0.62
82. " "	10	0.37	0.37	0.37	0.16	0.14	0.34	0.16	0.16	0.16	0.19	0.13	2.31	2.64	1.10	1.00	0.88	2.12	0.57
20. Splanchnoptosis	50	0.34	0.43	0.25	0.27	0.20	0.34	0.22	0.25	0.19	0.17	0.01	1.26	2.15	0.74	1.23	0.80	1.80	0.74
Men.																			
13. Carcinoma, anemia	28	0.36	0.41	0.23	0.24	0.20	0.34	0.20	0.21	0.16	0.17	0.00	1.50	2.05	0.70	1.20	0.98	2.12	0.68
14. Pernicious anemia	11	0.42	0.42	0.42	0.15	0.12	0.39	0.15	0.15	0.15	0.25	0.14	2.80	3.50	1.08	1.00	0.80	2.60	0.60
103. Hemophilia	34	0.39	0.35	0.47	0.27	0.14	0.50	0.17	0.17	0.17	0.17	0.12	1.44	2.41	0.94	1.60	0.85	2.94	0.56
109. "	38	0.37	0.37	0.37	0.27	0.16	0.45	0.19	0.19	0.19	0.17	0.06	1.37	2.31	0.82	1.42	0.84	2.37	0.61
87. Addison's disease	48	0.46	0.52	0.40	0.31	0.20	0.43	0.21	0.24	0.18	0.26	0.10	1.80	2.60	0.93	1.49	0.83	2.39	0.82

DISCUSSION.

Lipoids of Normal Blood.—For reasons discussed in the earlier part of the paper a detailed comparison of the normal values recorded above with those in the literature is not considered profitable. The "total fatty acids" and "total ether-soluble" values fall within the wide limits given in the literature. The "lecithin" is higher than the values given in the earlier literature but is about the same as that more recently reported.[67] The cholesterol values are higher than most of the reported values (which average about 0.17 gm. per 100 cc.),[34] a fact that is partly at least accounted for by the method used which, as has been noted before,[60] gives higher values than the methods in common use.

The variations from the average of the various constituents are from 10 to 20 per cent, being greatest for "total fatty acids" and least for "lecithin."

The differences in the content of lipoids of men's and women's blood is not great, and it is possible that a more extended series of analyses may eliminate these differences. The total fatty acids of the women's plasma is slightly higher than that of the men, while that of the corpuscles is somewhat lower. The same is true of the glycerides. Cholesterol and "total ether-soluble" are practically the same in both. "Lecithin" is lower in the plasma and higher in the corpuscles of the women than of the men.

The "lecithin" content of the corpuscles is always greater than that of the plasma, approximately double, while the cholesterol and total fatty acid content of the corpuscles is about the same, although generally a little less.

The value for "lecithin" in the corpuscles is generally about twice that of cholesterol, $\frac{L.}{C.} = 2.08$, average, in men and 2.14 in women, while in the plasma the values for the two constituents are nearly equal, $\frac{L.}{C.} = 0.96$ for men and 0.82 for women. The constancy of these ratios, which holds also for most of the pathological blood (see below), suggests a definite relationship between these substances.

In the blood both of men and women the normal cholesterol value is about one-third that of the "total ether-soluble." The lecithin is about the same fraction of the "total ether-soluble" in men's blood but is a little lower, about one-fourth of this value in women's blood.

The value for "fat" (which represents all the fatty acid not combined as lecithin) in the corpuscles is generally zero in women's blood and frequently so in men's, making it probable that all the cholesterol of the corpuscles is free (not combined with fatty acid). The absence of fat from the corpuscles raises the question whether there is any present in the normal plasma. Letsche[6] found none. The insolubility of fat in water and the fact that such a small amount of suspended fat is needed to make water cloudy while normal plasma is almost always clear would seem to give some support to this finding. On the assumption made above that two-thirds of the cholesterol of the plasma is combined, the amounts of glycerides in the plasma are small. If we assume further, as Klemperer[7, 21] has done, that all the cholesterol in the plasma is combined the glycerides will be reduced to amounts which are probably not far from the limit of error of the determinations. This is a point of considerable importance in determining the significance of lipemia (milkiness of the plasma) in pathological conditions, also in determining how the fat is normally transported in the blood, especially in fasting, when considerable quantities of fat are passing and yet the plasma remains clear. It seems unlikely, on the other hand, that there is no free cholesterol in the plasma, for if that were the case it would be difficult to explain the action of cholesterol as an antihemolytic against several agents[68] which appear to react only with free cholesterol.

Lipoids of Pathological Blood.

A. Summary of Important Data Regarding the Individual Samples.

1. Mitral Stenosis.—Total fatty acids increased, correspondingly increased values for "fat," "total ether-soluble," and $\frac{\text{T.F.A.}}{\text{L.}}$.

17, 47, 2, 70. Diabetes.—Nos. 17 and 47 practically normal (cholesterol low in 17). In Nos. 2 and 70, all the constituents

were much increased, although the increases in total fatty acids are relatively less than in lecithin and cholesterol. The plasma in Case 47 was somewhat cloudy; in all the others it was clear.

3. Debility.—"Total fatty acids," "fat," and the ratio $\frac{\text{T.F.A.}}{\text{L.}}$ slightly high, especially in the corpuscles. Lecithin and $\frac{\text{L.}}{\text{C.}}$ in corpuscles slightly increased.

4. Carcinoma of Stomach, Anemia.—"Total ether-soluble" high, "total fatty acids" and cholesterol high in corpuscles, "lecithin" low in corpuscles, "fat" high in both. Marked disturbance of the ratios $\frac{\text{T.F.A.}}{\text{L.}}$ and $\frac{\text{L.}}{\text{C.}}$ in the corpuscles.

12. Carcinoma of Peritoneum, Transverse Colon, Uterus, and Stomach.—Blood lipoids practically normal. Increase in cholesterol in corpuscles.

6. Carcinoma of Stomach, Hernia of Abdominal Wall, Hematuria.—Lipoids practically normal. Lecithin somewhat low, especially in corpuscles.

7. Chronic Myocarditis, Hypertension.—No marked disturbance in the distribution of the lipoids. "Total fatty acids" relatively lower in plasma and higher in corpuscles than normal.

8. Malnutrition.—"Total fatty acids" and "fat" low in plasma and high in corpuscles. "Total ether-soluble" low.

9. Chronic Nephritis, Chronic Myocarditis, Hypertension.—"Total ether-soluble" high; "total fatty acids" and "fat" high in both plasma and corpuscles. "Lecithin" increased also, but to a less extent. The ratios, however, fall within the limits of normal variation. Plasma cloudy.

10. Syphilis, Chronic Alcoholism.—"Total fatty acids," "fat," and cholesterol high in corpuscles; "lecithin" and cholesterol low in plasma. "Total ether-soluble" low. $\frac{\text{T.F.A.}}{\text{L.}}$ in corpuscles double the normal value. Plasma cloudy.

11. Goiter, Hyperthyroidism (?).—Lipoids normal.

5. Bronchial Asthma (?).—"Total fatty acids" and "fat" high, resulting in a high $\frac{\text{T.F.A.}}{\text{L.}}$ ratio.

15. Anemia Due to Bothriocephalus Latus.—"Lecithin" low throughout; cholesterol very low in plasma. "Fat" high in both plasma and corpuscles. The ratio $\frac{\text{T.F.A.}}{\text{L.}}$ is very high throughout; $\frac{\text{L.}}{\text{C.}}$ in the corpuscles is low.

16. Pernicious Anemia.—Cholesterol low in plasma. Lecithin low in corpuscles. Plasma slightly cloudy.

21. Pernicious Anemia, Hemorrhagic Stomatitis.—Cholesterol and "lecithin" low in plasma. "Total fatty acids" high in corpuscles, and relatively high in plasma. $\frac{\text{T.F.A.}}{\text{L.}}$ high in both corpuscles and plasma.

82. Pernicious Anemia.—Lecithin and cholesterol low throughout. Plasma cloudy.

20. Splanchnoptosis.—Lipoids practically normal. Plasma milky. (This blood was not obtained until it was 24 hours old.)

13. Anemia (?), Carcinoma of Stomach (?).—"Lecithin" low throughout.

14. Syphilis, Pernicious Anemia.—"Total fatty acids" slightly high. "Lecithin" very low in plasma, cholesterol low in plasma and corpuscles. "Fat" high in plasma. $\frac{\text{T.F.A.}}{\text{L.}}$ high throughout.

103 and 109. Hemophilia.—"Lecithin" low in plasma. Cholesterol also low, but little if any beyond the bounds of normal variation.

87. Addison's Disease.—Lipoids practically normal. Fat slightly high in plasma. Total ether-soluble high but within the normal limits.

Of these samples the following are practically normal as regards both the amounts and distribution of the blood lipoids: Diabetes (mild) (17 and 47); carcinoma of peritoneum (12); carcinoma of stomach (6); chronic myocarditis (7); goiter (11); splanchnoptosis (20); Addison's disease (87).

B. Summary of Data Regarding the Individual Lipoids.

"Total Fatty Acids."

Increased.—Mitral stenosis (1) (corpuscles); diabetes (2 and 70); debility; chronic myocarditis (7) (corpuscles); malnutrition (8)

(corpuscles); chronic nephritis (9); alcoholism (10); bronchial asthma (5); carcinoma of stomach (4); pernicious anemia (21), pernicious anemia (14), Addison's disease (87) (plasma).

Decreased.—Chronic myocarditis (plasma).

"Lecithin."

Increased.—Diabetes (2 and 70) (plasma); chronic nephritis (9) (corpuscles).

Decreased.—Diabetes (17) (plasma); carcinoma, anemia (4) (corpuscles); carcinoma, hemophilia (6); anemia (15); pernicious anemia (16) (corpuscles); pernicious anemia (21) (plasma); pernicious anemia (82); carcinoma, anemia (13) (plasma); syphilis, pernicious anemia (14) (plasma); hemophilia (103 and 109) (plasma).

Cholesterol.

Increased.—Diabetes (2) (plasma), (70) (plasma and corpuscles); carcinoma, anemia (4) (corpuscles); carcinoma of peritoneum (12).

Decreased.—Diabetes (17) (plasma and corpuscles); malnutrition (8) (corpuscles). Syphilis, alcoholic (10) (plasma); bronchial asthma (5) (plasma); anemia (15) (plasma); pernicious anemia (82) (plasma and corpuscles); syphilis, anemia (14) (plasma and corpuscles); hemophilia (103) (plasma).

"Fat."

High.—Mitral stenosis (1) (corpuscles); diabetes (47) (plasma); debility (3) (plasma); carcinoma, anemia (4) (plasma and corpuscles); carcinoma, hemophilia (6) (plasma and corpuscles); chronic nephritis (9) (plasma and corpuscles); syphilis, alcoholic (10) (corpuscles); bronchial asthma (5) (plasma and corpuscles); anemia (15) (plasma and corpuscles); anemia (16) (plasma); anemia (21) (corpuscles); anemia (82) (corpuscles); Addison's disease (87) (plasma).

Low.—Carcinoma of peritoneum (12) (plasma); malnutrition (8) (plasma). "Total ether-soluble" is outside the normal limits of variation in only five cases. It is high in diabetes (2 and 70), carcinoma (4), and chronic nephritis (9); low in alcoholism (10).

C. Discussion of Lipoids of Pathological Blood.

The constituent most often abnormal in value is the "fat" which is increased in fifteen and decreased in two of the twenty-five samples examined. The next in frequency of variation is the "lecithin," decreased in thirteen and increased in three. Then come the "total fatty acids," increased in twelve and decreased in one; then cholesterol which is increased in four cases and decreased in nine; and finally the "total ether-soluble" which is outside the normal limits of variation in only five samples.

Abnormal values for cholesterol and "lecithin" occur most frequently in the plasma—the composition of the corpuscles tending to remain constant with regard to these constituents. Variations of "total fatty acids" and "fat" occur with almost equal frequency in plasma and corpuscles. The increases in "total fatty acids" in general result from increases in "fat," the notable exceptions being the diabetics (2 and 70) where the "lecithin" and presumably also the cholesterol esters contribute. The increase of "total fatty acids" in the corpuscles is relative as well as absolute, the ratio $\frac{\text{T.F.A.}}{\text{L.}}$ being high in fifteen out of the twenty-five samples. The low lecithin values in many cases also increase the value of the ratio. In the plasma the ratio $\frac{\text{T.F.A.}}{\text{L.}}$ is abnormal in eleven samples.

The ratio $\frac{\text{L.}}{\text{C.}}$ shows frequent abnormality in the corpuscles but is remarkably constant in the plasma, being outside the normal figures in only six cases out of the twenty-five. The constancy of the ratio suggests strongly a close relationship between the two. "Lecithin" has been found to increase during fat absorption by all who have investigated this point,[27] and "lecithin" therefore is definitely indicated as a step in fat metabolism. Cholesterol has been reported to increase similarly, and although this finding has not been corroborated by the author's recent work (at least during the first 8 hours after feeding), the constancy of the relationship $\frac{\text{L.}}{\text{C.}}$ which persists in pathological conditions suggests strongly that cholesterol (as its esters ?) also has a part in fat metabolism, although presumably at a later stage than "lecithin."

The above results indicate that the most characteristic features of pathological conditions are the increase in the "fat" of plasma and corpuscles and decrease of "lecithin" of the plasma. These are particularly interesting in view of the above finding that lecithin is probably a stage in the metabolism of the fats and that the lecithin formation takes place in the corpuscles, and would seem to indicate a diminished activity of this mechanism in diseased conditions. Where the increase in fat is not accompanied by abnormalities in the lecithin values, it might be interpreted as the result of increased mobilization.

Low cholesterol values occur almost always in those conditions where the vitality is low, as in blood samples 8, 10, 15, 82, 14, a finding which is in agreement with those reported in the literature.[29, 46, 48] In this connection the recent work of McCrudden[71] is interesting. In a case of muscular weakness which he studied he found a relation between the severity of the condition and the low values for cholesterol and blood sugar. As the patient improved under treatment, the values for both blood sugar and cholesterol increased. Whether there is any connection between the cholesterol and blood sugar could of course only be settled by extended study, but the fact that the cholesterol is high in severe diabetes (70) where the blood sugar is also high (0.26 per cent) is suggestive.

With such limited data only the most general inferences can be made regarding individual conditions. In mild diabetes (17 and 47, urine free from sugar and diacetic acid) the values for the blood lipoids are normal or below normal throughout and with no notable variations from the normal ratios. In severe diabetes (2 and 70) in relatively good clinical condition (urine containing small amounts of sugar but no diacetic acid) all the blood lipoids are increased—up to 100 per cent or more, but the ratios are again not notably disturbed. In none of these cases was there any lipemia (in the sense of cloudy or milky appearance of the plasma) in spite of the high lipoid values. The constancy of the ratios with the lack of visible fat in the plasma would seem to indicate that in diabetes under good treatment the fat-utilizing mechanism is functioning in a practically normal manner, although requiring in the severe cases a high lipoid "pressure" to keep it going. The increase of "lecithin" and cholesterol along with the

fat is in agreement with the findings of Fischer[17] and Klemperer[7, 21] in diabetes.

In anemia the low values for cholesterol in the plasma are not without significance, in view of the part which cholesterol is said to play in protecting the corpuscles from the action of hemolytic agents, as suggesting a possible reason for the destruction of the corpuscles in these conditions.

In hemophilia the low values for "lecithin" are suggestive in view of the connection of cephalin with blood clotting shown by Howell[69] and the more recent observations of Hurwitz and Lucas[70] that cephalin injected into the circulation increases the speed of clotting, also that local applications of cephalin to wounds quickly stops bleeding.

SUMMARY.

The term "lipoids" is used in this paper as a general term for all those substances connected with the metabolism of the fatty acids, including the fatty acids, their naturally occurring compounds, and such substances as cholesterol which occur naturally in combination with the fatty acids and which are therefore presumably connected with their metabolism. Reasons for the choice of the term are given.

Complete analyses of the lipoids of the blood of twenty-three normal and twenty-five abnormal persons are presented, together with ratios between certain of these lipoids. The more important results are as follows:

Normal Blood.—The "lecithin" content of the corpuscles is found to be approximately double that of the plasma, while the cholesterol and "total fatty acid" values are almost always lower in the corpuscles than in the plasma. The value for "lecithin" in the corpuscles is generally about twice that of cholesterol, while in the plasma their values are nearly equal. The ratio between these constituents($\frac{L.}{C.}$) is quite constant in normal blood (especially plasma) and remains so in most of the pathological samples, suggesting a definite relationship between these constituents, and making it probable that cholesterol (as its esters?) has a part in fat metabolism.

In the blood both of men and women the normal cholesterol value is about one-third that of the "total ether-soluble." The

"lecithin" value is about the same fraction in men but is a little lower (one-fourth) in women. The amount of fat (glycerides) present in normal plasma is small. In the corpuscles it may be entirely absent.

Pathological Blood.—The blood lipoids are normal throughout in only eight of the twenty-five samples, while the "total ether-soluble" (representing what is ordinarily termed "fat") is within normal limits in twenty out of the twenty-five. The importance of the complete lipoid analysis in investigating abnormal conditions as against the ordinary determination of "fat" is thus emphasized.

The most characteristic feature of pathological conditions in human beings is the increase of "total fatty acids" and "fat" both in plasma and corpuscles, and the decrease of "lecithin" in the plasma. Since the "fat" is probably to be regarded as the inactive form of the body lipoids, the form in which they are stored—the raw material of fat metabolism—and the "lecithin" as the first step in its utilization, an undue accumulation of "fat" or a notably decreased value for "lecithin" probably indicates a diminished activity of the fat metabolism. The ratio $(\frac{L.}{C.})$ in the plasma is constant and normal in all but six of the pathological samples.

In severe diabetes the blood lipoids are all greatly increased but the ratios between these constituents are practically normal. In mild diabetes the blood lipoids are practically normal.

The low values for the cholesterol of the plasma in anemia are suggestive in view of the antihemolytic function ascribed to cholesterol.

The low values for "lecithin" (which includes cephalin) in the plasma of hemophilia are noteworthy in view of the connection recently shown between cephalin (thromboplastic substance of Howell) and blood coagulation.

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THE EFFECT ON NITROGEN PARTITION OF SUBSTITUTING ALCOHOL FOR SUCROSE IN AN OTHERWISE FIXED DIET.

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INTRODUCTION.

In view of the great number of published reports on the physiological effect of alcohol, any further effort may seem superfluous. As far as I have been able to determine, however, there has never been published a report of the effect on nitrogen partition of substituting for sucrose an isodynamic amount of alcohol during an otherwise fixed diet. That alcohol under certain conditions causes a diminution in the total nitrogen excretion, in others an increase, and in some cases no change at all, is well known, and has been fully discussed by Atwater.[1]

Salant and Hinkel[2] were the first to report detailed results of the effect of alcohol on the composition of urine. They used dogs exclusively as subjects and studied the problem from the standpoint of adding alcohol to the diet. They determined the amount and ratios present of total nitrogen, phosphates, chlorides, and the various forms of sulfur.

In the same year Mendel and Hilditch[3] published the results of their work on the effect on nitrogen partition of adding alcohol to an otherwise fixed diet. They used men and dogs as subjects. They determined the total nitrogen, urea, ammonia, uric acid, purine-base, creatine, and creatinine nitrogen.

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These two series of investigations admirably complete the study of the effect of alcohol on the composition of the urine when the alcohol is added to the diet. However, they leave undetermined the effect on nitrogen partition of substituting isodynamic quantities of alcohol for sucrose during an otherwise fixed diet.

EXPERIMENTAL PROCEDURE.

The subjects used in these experiments were two men, A and B. Both subjects were not unaccustomed to the mild use of alcoholic beverages. The diet was the same for both and consisted of the following foods in the amounts indicated. As far as possible the same grade of food was consumed by both subjects throughout.

Breakfast, 7 a. m.		Lunch, 12 m.		Dinner, 6 p. m.	
	gm.		gm.		gm.
Grapenuts	30	Bread	150	Potato	100
Orange juice	250	Butter	30	Steak	100
Bread	50	Ham	33	Corn (canned)	100
Butter	15	Apple pie	100	Bread	50
Sugar	30	Sugar	30	Butter	30
Whole milk, cc.	325			Sugar	30

Water 2,000 cc. per day for A and 1,500 cc. per day for B.

Using Locke's[4] tables as a basis of calculation this diet yields about 2,800 calories and contains about 14 gm. of nitrogen.

At the beginning of the experiment A weighed 50 kg. At its termination he weighed 51 kg. With A, the experiment was divided into three periods, a preliminary period of 4 days and a terminal period of 3 days during which the diet was as given above, and an intermediate period of 4 days' duration. The dietary change consisted in substituting 40 cc. of rum for the 30 gm. of sucrose in each meal.

At the beginning of the experiment B weighed 70 kg. At its termination his weight was the same. With B the experiment

[4] Locke, E. A., Food Values, New York, 1915.

was likewise divided into three periods, consisting in this case of preliminary and terminal periods of 3 days each, during which the diet was as given above, and the intermediate period of 3 days' duration, the dietary change being the same as for A.

The total nitrogen, ammonia, uric acid, creatinine, and creatine were determined according to the methods of Folin and his collaborators. The urea was determined according to Marshall.[5]

RESULTS.

The ingestion of the alcohol produced a pharmacological response consistent with the quantity and individual tolerance.

The analytical results are shown in Tables I and II. The quantities noted represent the amounts of the various nitrogenous constituents excreted per 24 hours.

TABLE I.

Composition of Urine of A.

			Nitrogen partition.						In terms of total N.				
Date.	Volume.	Alcohol (45 per cent).	Total N.	Urea N.	NH_3 N.	Creatinine N.	Uric acid N.	Undetermined N.	Urea N.	NH_3 N.	Creatinine N.	Uric acid N.	Undetermined N.
1916	*cc.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Mar. 14	1,730		8.98	6.87	0.32	0.41	0.14	1.24	76.5	3.6	4.6	1.6	13.7
" 15	1,720		9.29	6.98	0.34	0.44	0.14	1.39	75.1	3.7	4.7	1.5	15.0
" 16	2,000		9.66	7.57	0.34	0.43	0.12	1.20	78.3	3.5	4.4	1.2	12.6
" 17	2,050		9.43	7.10	0.33	0.44	0.13	1.43	75.2	3.5	4.5	1.4	15.4
" 18	1,760	120	9.69	7.12	0.29	0.41	0.13	1.74	73.5	3.0	4.2	1.3	18.0
" 19	2,000	120	9.17	7.21	0.30	0.45	0.14	1.07	78.6	3.3	4.9	1.5	11.7
" 20	1,860	120	9.30	7.21	0.31	0.42	0.14	1.23	77.5	3.3	4.6	1.5	13.2
" 21	1,820	120	9.18	7.24	0.29	0.43	0.12	1.10	78.8	3.2	4.6	1.3	12.1
" 22	1,720		9.05	7.02	0.29	0.42	0.13	1.19	77.6	3.2	4.7	1.4	13.1
" 23	1,820		9.10	7.64	0.29	0.41	0.12	0.64	83.9	3.2	4.5	1.3	7.1
" 24	1,850		9.22	7.26	0.29	0.42	0.14	1.11	77.7	3.2	4.6	1.6	12.9

[5] Marshall, E. K., Jr., *J. Biol. Chem.*, 1913, xv, 487.

TABLE II.

Composition of Urine of B.

Date.	Volume.	Alcohol (45 per cent).	Nitrogen partition.						In terms of total N.				
			Total N.	Urea N.	NH_3 N.	Creatinine N.	Uric acid N.	Undetermined N.	Urea N.	NH_3 N.	Creatinine N.	Uric acid N.	Undetermined N.
1916	*cc.*	*cc.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Mar. 29	1,110		9.43	7.54	0.47	0.43	0.12	0.87	79.9	4.9	4.6	1.3	9.3
" 30	1,340		9.64	7.44	0.45	0.44	0.13	1.18	77.2	4.7	4.6	1.3	12.2
" 31	1,270		9.33	7.60	0.50	0.44	0.12	0.67	81.5	5.4	4.8	1.3	7.1
Apr. 1	1,200	120	9.63	7.56	0.43	0.45	0.12	1.07	78.5	4.5	4.6	1.2	11.2
" 2	1,040	120	9.77	7.64	0.40	0.44	0.19	1.10	78.2	4.1	4.5	1.9	11.3
" 3	990	120	9.61	7.06	0.49	0.42	0.13	1.51	73.5	5.1	4.4	1.3	15.7
" 4	1,350		9.01	7.10	0.35	0.41	0.12	1.03	78.8	3.7	4.5	1.3	11.5
" 5	1,000		9.52	7.92	0.41	0.42	0.14	0.63	83.2	4.3	4.4	1.5	6.6
" 6	1,100		9.66	7.77	0.43	0.41	0.14	0.91	80.4	4.4	4.3	1.5	9.4

A study of these tables shows that the substitution of sucrose yielding about 370 calories by an isodynamic quantity of alcohol in an otherwise fixed diet produces no significant changes in either amount or ratios of the nitrogenous urinary constituents determined.

In Rosemann's[6] observations it was found that although alcohol may replace carbohydrate or fat, with an equivalent protein-sparing action, in many cases this effect did not appear until some time after the substitution of alcohol in the diet.

In these experiments alcohol apparently spares protein to the same extent as does an isodynamic quantity of sucrose, and this sparing effect is obtained immediately.

Creatine is not included in the tables because at no time could measurable amounts be obtained, thus confirming the results of Mendel and Hilditch.[3]

Landau,[7] Jackson,[8] Chittenden,[9] Mendel and Hilditch,[3] Beebe,[10]

[6] Rosemann, E., *Oppenheimer's Handb. Biochem.*, 1909–11, iv, pt. i, 413.
[7] Landau, A., *Deutsch. Arch. klin. Med.*, 1909, xcv, 280.
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and others found that the addition of alcohol to the diet resulted in an increased excretion of uric acid.

According to Mareš,[11] the amount of uric acid excreted depends largely upon the activity of the digestive glands. Hence increased activity of these glands, due either to additional work put upon them by increasing the ingesta, or to their stimulation through the pharmacodynamical action of the alcohol, would tend to an increase in uric acid output.

If the former possible cause be removed by the simple expedient of giving isodynamic diets, the one carrying sugar and the other alcohol, any changes obtained must be attributed to the pharmacological action of the alcohol. The tables show that no change in uric acid excretion resulted from this procedure.

This does not necessarily indicate that all or even the greater part of the increase in uric acid excretion obtained by Mendel[3] and others, when they added alcohol to the diet, was due to increased caloric intake.

In the first place, the amount of alcohol ingested by Mendel's subjects was nearly 40 per cent more than that consumed by mine. This would lead to a more extended pharmacodynamical action. In the second place the subjects used by Mendel were not accustomed to the use of alcohol as a beverage and hence might be expected to react to a greater extent than would my subjects, from whose continuous though mild use of alcohol we might expect the development of a tolerance.

Comparing our results with the statement of Mendel and Hilditch[3] that *even in the 'moderate' doses used alcohol increases the output of uric acid*, it is indicated that several factors contribute to the effect of alcohol on uric acid excretion, and that in the light of our present knowledge, we can make no statement as to the relative importance of any one of these factors, much less explain their action.

CONCLUSIONS.

In subjects accustomed to the moderate use of alcoholic beverages the substitution in an otherwise fixed diet of alcohol for sucrose in isodynamic amounts, yielding about 370 calories, produces no significant changes in urinary nitrogen partition.

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VICINE AND DIVICINE.

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Plate 6.

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Recent developments in the chemistry of the nucleic acids have renewed the interest of chemists in vicine, discovered by Ritthausen[1] over 40 years ago. Schulze and Trier,[2] on the basis of Ritthausen's analytical data, were able to recognize in vicine a pyrimidine glucoside. As such, the substance resembled the pyrimidine nucleosides entering into the composition of nucleic acid. As a hexoside, it had a possible bearing on the nucleic acids of the animal type. The fact that plant nucleic acids containing hexoses have not yet been discovered, added still more mystery and interest to the subject; hence the simultaneous activity of several laboratories in solving the problem of the structure of vicine and divicine.

Chronologically, the first paper after that of Schulze and Trier was one by Treat B. Johnson.[3] Arguing from Ritthausen's divicine data and from personal experience with Traube's[4] 4,5-diamino-2,6-dioxypyrimidine, Johnson came to the conclusion that the two bases were identical. In the course of work on Traube's[5] diaminopyrimidines,

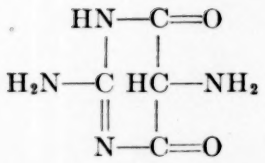
[1] Ritthausen, H., and Kreusler, U., *J. prakt. Chem.*, 1870, ii, 336. Ritthausen, H., *ibid.*, 1873, vii, 374; 1881, xxiv, 202; 1899, lix, 480. Ritthausen, H., and Preuss, *ibid.*, 487. Ritthausen, *Ber. chem. Ges.*, 1876, ix, 301; 1896, xxix, 894, 2108.

[2] Schulze, E., and Trier, G., *Z. physiol. Chem.*, 1910–11, lxx, 143.

[3] Johnson, T. B., *J. Am. Chem. Soc.*, 1914, xxxvi, 337.

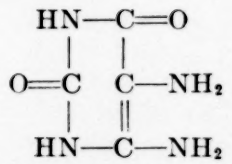
[4] Traube, W., *Ber. chem. Ges.*, 1900, xxxiii, 1382.

[5] Traube, *Ber. chem. Ges.*, 1893, xxvi, 2556.



2,5-Diamino-4,6-dioxypyrimidine.

and



4,5-Diamino-2,6-dioxypyrimidine.

Johnson and Johns[6] discovered two reactions. The first led to the formation of uric acid by fusion of the 4,5-diamino base with urea; the second enabled them to distinguish an amino group in the 5-position by its color reaction with molybdic acid solution. E. Fischer[7] used the first of the two Johnson reactions to test the structure of divicine, but found that, on fusion with urea, it behaved differently from the 4,5-diamino base. He also observed that the two bases differed in their water of crystallization. He was, however, so much impressed by the logic of Johnson's arguments that he accepted the 4,5-diamino-2,6-dioxypyrimidine structure for divicine, attributing the differences between the two substances to stereoisomerism. Thannhauser and Dorfmüller[8] also accepted Johnson's views and proceeded to prepare the nucleoside synthetically—they supposed, with success.

Preceding Fischer's publication by approximately 10 weeks, appeared a note on vicine by Levene.[9] He reported experimental facts which led him to conclude that divicine was the 2,5- not the 4,5-diamino base. His conclusions were drawn from the similarity between divicine and the 2,5-diamino base in respect to water of crystallization and behavior towards urea and towards nitrous acid. The 4,5-diamino base differs in all three points from both divicine and the 2,5-diamino base. In a private letter, Fischer accepted the conclusions of Levene.

However, the entire question of the structure of vicine and divicine did not seem definitely solved. The views of Levene as to the positions of the amino groups were based on analogy in the behavior of the molecules as wholes. Direct chemical evidence

[6] Johnson, T. B., and Johns, C. O., *J. Am. Chem. Soc.*, 1914, xxxvi, 545, 970.

[7] Fischer, E., *Ber. chem. Ges.*, 1914, xlvii, 2611.

[8] Thannhauser, S. J., and Dorfmüller, G., *Ber. chem. Ges.*, 1915, xlvii, 1304.

[9] Levene, P. A., *J. Biol. Chem.*, 1914, xviii, 305.

was lacking. The present investigation furnishes such evidence regarding the location of the two amino groups.

The presence of one amino group in the 2-position was demonstrated by the oxidation of divicine to guanidine. Many years ago Fischer[10] obtained guanidine on oxidizing 2-imidouric acid with potassium chlorate. It was expected that 2,5-diamino-4,6-dioxypyrimidine would yield guanidine under the same conditions. The expectation was realized, and thus direct proof was furnished of the presence of an amino group in the 2-position.

Until now, the color test of Johnson has been the only evidence for the location of the second amino group. This evidence was also substantiated by direct chemical proof. Both divicine and the synthetic 2,5-diamino base were converted into 2-imidopseudouric acid, which possessed a very definite crystalline form, and physical properties differing from those of the 4-imidopseudouric acid obtained from the 4,5-diamino base.

It was intended to convert both imidopseudouric acids into purine derivatives. 2-Imidouric acid was expected from one, uric acid from the other. The conversion into uric acid of 4-imidopseudouric acid was accomplished quite readily. Indeed this substance is probably an intermediate product in the uric acid synthesis of Johnson. The conversion into uric acid of 2-imidopseudouric acid, obtained from either divicine or the synthetic 2,5-diamino base, proved impractical under the conditions of the present work. The reactions, however, gave no uric acid.

Occasion was taken to repeat the condensation of 4,5-diamino-2,6-dioxypyrimidine with urea. In Levene's previous work, the substances prepared under the conditions described by Johnson and Johns, although quite granular and non-hygroscopic, were amorphous. Johnson and Johns made no reference to the crystalline character of their substance. Since a small amount of amorphous material had also been obtained by condensing urea with the 2,5-diamino base, it was concluded to make an effort to crystallize the substances formed by the condensation of each of the three bases with urea. Pure crystalline uric acid was obtained from the 4,5-diamino base, after the reaction product had been repeatedly dissolved in alkali and reprecipitated by the

[10] Fischer, *Ber. chem. Ges.*, 1897, xxx, 571.

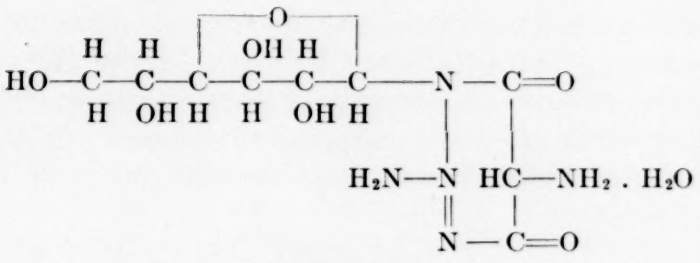
addition of acid. From the other two bases, it was very difficult or impossible to prepare a crystalline condensation product, and when it was obtained its crystalline form differed entirely from that of either uric acid or 2-imidopseudouric acid.

The composition of the sulfates was also redetermined, since the analytical data previously reported were unsatisfactory. In spite of many recrystallizations, it was not possible to get perfectly definite results. Apparently the sulfates underwent hydrolysis in the course of purification. Fischer, too, had arrived at the same conclusion regarding the sulfate of divicine. However, in a general way, the impression was substantiated that the sulfate of the 4,5-diamino base crystallizes with more water of crystallization than does that of the 2,5-diamino base, and that in this respect the sulfate of divicine more closely resembles the latter.

Hence, the structure of the base may be regarded as conclusively established. Divicine is 2,5-diamino-4,6-dioxypyrimidine.

Passing from the base to the whole vicine molecule, it appeared that even the most recent publications had left the data very incomplete. There remained discrepancies between the analytical values published by Ritthausen and the values required by the formulas suggested by either Johnson or Levene. Furthermore, Ritthausen had great difficulty in preparing different samples of vicine of constant composition. Perhaps on this account Fischer considered the possibility of its being a polynucleoside. An effort was therefore made to determine the conditions necessary to prepare perfectly pure vicine. These were attained when the substance was recrystallized from water and dried without driving off its single molecule of water of crystallization. Ritthausen, who was the only worker besides the authors to publish analyses of vicine, recrystallized his material from alcoholic solutions. Partial dehydration probably caused his irregular results.

In addition, the monomolecular nature of the nucleoside was demonstrated by molecular weight determinations. This evidence also shows that only one hexose and one pyrimidine are present in the molecule of vicine. The hexose was shown independently by Levene and by Fischer to be *d*-glucose. The following formula for vicine is therefore suggested.



Vicine.

There remain two points to be cleared up in regard to the structure of vicine; the particular atom of the pyrimidine base to which the sugar is joined in glucosidic union remains unknown, and the α or β nature of this glucosidic linkage is still to be determined. Work on these questions is now in progress.

EXPERIMENTAL PART.

Preparation of Vicine.—Only two slight changes were made in the method of preparing vicine described by Levene.[9] 3 per cent sulfuric acid was used instead of 5 per cent in the extraction of the vetch meal. The filtrate from the mercuric sulfide was concentrated to small volume, filtered, and cooled. The vicine crystallized out from the aqueous solution. The mother liquors were worked up for a further yield by the method of alcoholic extraction. In this way, a yield of about 2.5 gm. of vicine per kg. of vetch seeds was obtained. The substance was purified by recrystallizing it from water. Bone-black was used as a decolorizer, and the amount of water necessary was 20 cc. per gm. of vicine. Two such treatments gave a product that was almost snow-white and pure enough for all but analytical purposes.[11]

Analysis of Vicine.—Vicine contains one molecule of water of crystallization. At 100° *in vacuo* over sulfuric acid, it loses this water very slowly, at 135° the loss is much more rapid, and at 155° there is a slight discoloration before equilibrium is attained. Assuming the mononucleosidic formula, and adding to it one

[11] To obtain the lipoids from vetch seeds, Dr. C. J. West extracted the seeds several times with 95 per cent alcohol. This extract was fractionated, and one fraction found to consist largely of vicine. When purified by recrystallization from water, it proved to be identical with the material prepared as above.

molecule of water, the calculated analysis of vicine agrees very closely with the figures found. A sample of the substance prepared by the method described above was recrystallized four times from water, and dried by exposure to the air. When further dried at room temperature *in vacuo* over sulfuric acid, its weight remained constant.

0.1032 gm. substance gave 0.1401 gm. CO_2 and 0.0523 gm. H_2O.
0.1012 " " required 12.45 cc. of 0.1 N HCl.
0.0991 " " " 12.15 " " 0.1 " "

A portion of this material, when heated to 61° *in vacuo* over H_2SO_4, remained constant in weight. Another sample, when heated to 100° under the same conditions, lost weight very slowly. Two portions were then heated to constant weight *in vacuo* over sulfuric acid, and the percentage of water lost was determined.

I. This portion came to constant weight after 12 hours at 135°. There was no discoloration of the material. 0.5174 gm. substance lost 0.0285 gm. weight.

II. This portion came to constant weight after 18 hours at 155°. The material was very slightly discolored. 1.0177 gm. substance lost 0.0588 gm. weight.

	Calculated for $C_{10}H_{16}N_4O_7.H_2O$:	Found:	
C	37.24	37.02	
H	5.63	5.67	
N	17.39	17.24	17.18
H_2O	5.59	I. 5.51	II. 5.78

I. 0.1015 gm. dry substance gave 0.1442 gm. CO_2 and 0.0491 gm. H_2O.
0.1023 " " " required 13.35 cc. 0.1 N HCl.
0.1014 " " " " 13.10 " 0.1 " "
II. 0.1043 " " " gave 0.1489 gm. CO_2 and 0.0505 gm. H_2O.
0.1049 " " " required 13.50 cc. 0.1 N HCl.
0.1008 " " " " 13.15 " 0.1 " "

	Calculated for $C_{10}H_{16}N_4O_7$:	Found: I.		II.	
C	39.45	38.75		38.94	
H	5.30	5.41		5.42	
N	18.42	18.29	18.10	18.19	18.28

Molecular Weight of Vicine.—The molecular weight of vicine agrees with the mononucleosidic formula. The fact that the substance is monomolecular shows that there can be only one sugar

and only one pyrimidine group present in the unhydrolyzed compound. The sample used for the determinations was purified by repeated crystallization from water, and dried at room temperature *in vacuo* over sulfuric acid. The Beckmann boiling point method was used, with water as the solvent.

0.843 gm. substance in 19.46 gm. H_2O gave a rise of 0.070°.
0.498 " " " 19.49 " " " " " " 0.044°.

Assuming, on the basis of the analyses, that the substance contained 5.6 per cent water of crystallization,

0.796 gm. dehydrated substance in 19.51 gm. H_2O gave a rise of 0.070°.
0.470 " " " " 19.52 " " " " " " 0.044°.

	Calculated for $C_{10}H_{16}N_4O_7$:	Found:	
Mol. Wt.	304	302	284

Preparation of the Sulfates of the Three Bases.—These substances were found to have anomalous compositions. In order to recrystallize them, it was necessary to dissolve them in hot dilute solutions of sulfuric acid, and to precipitate them by adding alcohol to the cold, filtered solutions. The samples were air-dried. Even so, when this method was repeated several times in what seemed to be an exactly similar fashion, it was impossible to obtain sulfate of 2,5-diamino-4,6-dioxypyrimidine of constant composition. The analyses given are typical of the material used in the experiments later described.

4,5-Diamino-2,6-dioxypyrimidine Sulfate.—This substance was prepared by the method of Traube.[4] After several recrystallizations in the manner described above, a substance of constant composition was obtained.

0.1974 gm. substance gave 0.1671 gm. CO_2 and 0.0775 gm. H_2O.
0.1008 " " required 19.25 cc. 0.1 N HCl.
0.1000 " " " 19.15 " 0.1 " "
0.2024 " " gave 0.1048 gm. $BaSO_4$.
0.2007 " " " 0.1043 " "

	Calculated for $(C_4H_6N_4O_2)_2H_2SO_4.2H_2O$:	Calculated for $(C_4H_6N_4O_2)_2H_2SO_4.4H_2O$:	Found:	
C	22.95	21.13	23.09	
H	4.34	4.88	4.39	
N	26.80	24.65	26.75	26.83
H_2SO_4	23.45	21.59	21.76	21.84

2,5-Diamino-4,6-dioxypyrimidine Sulfate.—This substance was prepared by the method of Traube,[5] and recrystallized several times in the manner described above.

a. 0.1056 gm. substance required 21.10 cc. 0.1 N HCl.
b. 0.0990 " " " 19.80 " 0.1 " "

The substance was recrystallized under conditions apparently exactly similar to the previous ones.

c. 0.1009 gm. substance required 20.75 cc. 0.1 N HCl.
d. 0.0980 " " " 20.20 " 0.1 " "

	Calculated for $(C_4H_6N_4O_2)_2H_2SO_4.0.5H_2O$:	Calculated for $(C_4H_6N_4O_2)_2H_2SO_4.H_2O$:	Found:
N.....	28.65	28.00	a. 27.99 b. 28.02 c. 28.83 d. 28.88

Divicine Sulfate.—5 gm. of vicine were placed with 10 cc. of 20 per cent sulfuric acid in an unstoppered Erlenmeyer flask which was then immersed in boiling water. The vicine dissolved within a minute or two. After 8 minutes' heating a crystalline precipitate began to form, and at the end of 15 minutes the contents of the flask were a thick paste. The flask was removed, its contents were diluted with alcohol, and the vessel was cooled in ice. The precipitate was filtered out and dried. The yield was about 3 gm. No attempt was made to obtain this substance of constant composition. After recrystallization in the manner described above, a sample analyzed as follows:

0.1024 gm. substance gave 0.0934 gm. CO_2 and 0.0350 gm. H_2O.
0.1000 " " required 19.25 cc. 0.1 N HCl.
0.0999 " " " 19.30 " 0.1 " "
0.3014 " " gave 0.1809 gm. $BaSO_4$.
0.3009 " " " 0.1799 " "

	Calculated for $(C_4H_6N_4O_2)_2H_2SO_4.0.5H_2O$:	Calculated for $(C_4H_6N_4O_2)_2H_2SO_4.2H_2O$:	Found:	
C.....	24.53	22.95	24.89	
H.....	3.86	4.34	3.82	
N.....	28.65	26.80	26.97	27.07
H_2SO_4.....	25.07	23.45	25.22	25.12

Oxidation of the Sulfates.—The oxidation of the sulfates was carried out by a method analogous to the one used by Fischer[10] in the oxidation of 2-imidouric acid. 2.4 gm. of the sulfate

were placed in a bottle with 18 cc. of hydrochloric acid solution made up of equal volumes of acid of sp. gr. 1.19 and of water. The bottle was shaken for 2 hours. During the first hour and a half, 0.5 gm. of potassium chlorate was added to the mixture. In no case did complete solution occur. The reaction mixtures were filtered and cooled. With the sulfate of the 4,5-diamino base, no precipitation occurred. With the sulfates of the 2,5-diamino base and divicine, crystalline precipitates formed. These were filtered out. All three solutions were then evaporated to dryness *in vacuo*. The solid residues were taken up with alcohol, and the insoluble materials filtered out. Then the alcoholic filtrates were again evaporated to dryness *in vacuo*. The solid residues were dissolved in a few cc. of water, made alkaline with a little sodium hydroxide, and the solutions tested for the presence of guanidine.

$KClO_3$ and 4,5-Diamino-2,6-dioxypyrimidine Sulfate.—Sodium picrate was added to the solution obtained from the sulfate of 4,5-diamino-2,6-dioxypyrimidine by the method just described. There was no precipitate. Guanidine is therefore not formed in the reaction.

$KClO_3$ and 2,5-Diamino-4,6-dioxypyrimidine and Divicine Sulfates.—Sodium picrate was added to the solutions obtained from the sulfates of 2,5-diamino-4,6-dioxypyrimidine and divicine by the method just described. In each case a yellow precipitate appeared. These precipitates were filtered out and washed on the filter with water and ether. They were then dissolved in boiling water, and a little bone-black was added. The solutions were filtered and cooled. The precipitates which formed had the crystalline form found by Emich[12] to be characteristic of guanidine picrate. Both precipitates were recrystallized from alcohol and dried *in vacuo* over sulfuric acid at 106°.

a. Substance from 2,5-diamino-4,6-dioxypyrimidine sulfate.
0.0864 gm. substance gave 22.2 cc. of N at 26.1° and 759 mm.
b. Substance from divicine sulfate.
0.0868 gm. substance gave 22.05 cc. N at 26.4° and 766.5 mm.

	Calculated for $C_7H_8N_6O_7$:	Found:
N.....	29.18	a. 29.28 b. 29.21

[12] Emich, F., *Monatsh. Chem.*, 1892, xii, 23.

These figures are considered to prove that the substance is in both cases guanidine picrate.

The formation of guanidine picrate from divicine and 2,5-diamino-4,6-dioxypyrimidine, and its non-formation from 2,6-dioxy-4,5-diaminopyrimidine show that divicine has an amino group in the 2-position.

Treatment of the Sulfates with Urea.—This work is a repetition and confirmation of the work of Johnson and Johns[6] and Fischer.[7] In each case, 1 gm. of the sulfate was triturated with 1.5 gm. of urea and heated in a hard glass test-tube for 1 hour at 160–170°. The melt was then cooled and dissolved out with water. In every case the reaction mixture contained some insoluble material.

Urea and 4,5-Diamino-2,6-dioxypyrimidine Sulfate.—To the reaction mixture obtained from 2 gm. of material by the method described above, potassium hydroxide was added until the solid was nearly all dissolved. The solution was then acidified with acetic acid. A heavy amorphous precipitate appeared. This was filtered out, dissolved in 200 cc. of hot dilute potassium hydroxide, and poured into 250 cc. of hot 6 per cent hydrochloric acid. The precipitate formed was perfectly crystalline. It was filtered out, washed with water and alcohol, and dried *in vacuo* over sulfuric acid. The yield was 1.1 gm. The substance gave the murexide test with nitric acid, and formed with silver nitrate a white complex insoluble in ammonia.

0.1613 gm. substance gave 0.2115 gm. CO_2 and 0.0380 gm. H_2O.
0.1003 " " required 23.85 cc. 0.1 N HCl.

	Calculated for $C_5H_4N_4O_2$:	Found:
C	35.69	35.76
H	2.40	2.64
N	33.34	33.31

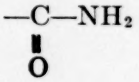
These results show that the substance is uric acid.

Urea and Divicine and 2,5-Diamino-4,6-dioxypyrimidine Sulfates.—In both cases a little acid was added to the turbid reaction mixtures obtained by the method described above, with the effect of increasing the amounts of precipitate. These were filtered out, boiled with bone-black in a little water, and the solutions filtered and cooled. The precipitates formed were filtered out and each dissolved in a little boiling water. In each case, the

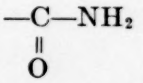
filtered solution gave a very slight precipitate on cooling. In the case of the substance from 2,5-diamino-4,6-dioxypyrimidine sulfate, this precipitate, although crystalline, did not in the least resemble the characteristic needles of 2-imidopseudouric acid. In the case of the substance from divicine sulfate the final precipitate was completely amorphous. No trace of uric acid was observed in either case.

The formation of uric acid by fusion with urea strikingly differentiates the sulfate of the 4,5-diamino base from the sulfates of divicine and the 2,5-diamino base.

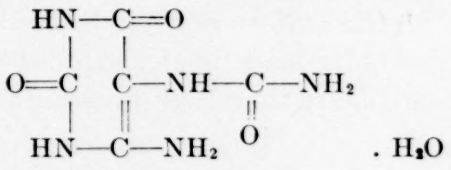
Preparation of Imidopseudouric Acids from the Sulfates.—Both of the synthetic sulfates, on being treated with potassium cyanate, yielded an imidopseudouric acid crystallizing with one molecule of water. One of them, the 2-imidopseudouric acid made from the 2,5-diamino base, has already been described by Traube;[13] the other, the 4-imidopseudouric acid from the 4,5-diamino base, is reported here for the first time. Analysis showed that the new compound contained a molecule of water of crystallization and was isomeric with the one described by Traube. Its conversion into uric acid (to be described later) showed that the



group must be attached to the amino group in either the 4- or the 5-position. Since the compound, like Traube's imidopseudouric acid, gave no blue color with molybdic acid in alkaline solution (Johnson's test for an amino group in the 5-position), it was concluded that the



group was attached to the amino group in the 5-position, and the following structural formula was assigned to the compound:



[13] Traube, *Ber. chem. Ges.*, 1893, xxvi, 2558.

The differences noted between the two substances were as follows:

2-Imidopseudouric Acid.	*4-Imidopseudouric Acid.*
1. Crystallized in long needles (Fig. 1).	Crystallized in oblong plates (Fig. 2).
2. At 100°, 1 liter of water dissolved 1.5 gm.	At 100°, 1 liter of water dissolved 7 gm.
3. Lost its water of crystallization in 8 hours at 135° *in vacuo* over sulfuric acid.	Lost its water of crystallization in 40 hours at 135° *in vacuo* over sulfuric acid.

In all three respects, the substance obtained from the sulfate of divicine (Fig. 3) was identical with the substance obtained from the sulphate of the 2,5-diamino base. This evidence confirms the view that divicine has an amino group in the 5-position. At the same time, certain differences in behavior between the sulfate of divicine and that of the 2,5-diamino base were noted.

Sulfate of the 2,5-Diamino Base.	*Sulfate of Divicine.*
1. When this substance was poured into boiling KOCN solution, a marked purple color appeared, which only vanished after being boiled some 10 minutes.	When this substance was poured into boiling KOCN solution, only faint and fugitive traces of purple color could be observed.
2. The yield of 2-imidopseudouric acid obtained from the above reaction was about 0.55 gm. per gm. of sulfate used.	The yield of 2-imidopseudouric acid obtained from the above reaction was about 0.04 gm. per gm. of sulfate used.

Concerning the cause of these differences no explanation can be offered. The method of preparing the imidopseudouric acid was the same in all three cases. 2 gm. of sulfate were poured into a boiling solution of 10 gm. of potassium cyanate in 20 cc. of water. After about 2 minutes' boiling, 4 gm. of solid potassium cyanate were added and the boiling was continued for 10 minutes more, or, in the case of the sulfate of the synthetic 2,5-diamino base, until the purple color had vanished. Then the hot reaction mixture was poured into an excess of hot 5 per cent hydrochloric acid.

1. 4-Imidopseudouric Acid from the Sulfate of the 4,5-Diamino Base.—When the hot acid solution, obtained as described above,

was cooled, a crystalline precipitate, only slightly contaminated by amorphous material, appeared at once. This was filtered out and dried. The yield was about 0.45 gm. per gm. of sulfate used. The substance was then boiled with bone-black in water, using about 135 cc. of water per gm. of material. When the filtered solution was cooled, the substance crystallized out almost colorless. This product was recrystallized four times from water, the hot solution being filtered each time.

2. 2-Imidopseudouric Acid from the Sulfate of the 2,5-Diamino Base.—When the hot acid solution, obtained as described above, was cooled, a crystalline precipitate, only slightly contaminated by amorphous material, appeared at once. This was filtered out and dried. The yield was about 0.55 gm. per gm. of sulfate used. The substance was then boiled with bone-black in water, using about 650 cc. of water per gm. of material. When the filtered solution was cooled, the substance crystallized out snow-white. This product was recrystallized twice from water, the hot solution being filtered each time.

3. 2-Imidopseudouric Acid from the Sulfate of Divicine.—When the hot acid solution, obtained as described above, was cooled, the crystalline precipitate only appeared after several days' standing, and was then contaminated by much amorphous impurity. The substance was filtered out and dissolved in boiling water, using about 650 cc. per gm. of material. The precipitate formed on cooling was filtered out, and the process repeated once or twice more until the pure white material showed no trace of amorphous impurity. The substance was then filtered out and dried. The yield was about 0.04 gm. per gm. of sulfate used. This product was recrystallized four times from water, the hot solution being filtered each time.

In all three cases, the final materials were dried *in vacuo* over sulfuric acid at room temperature. No one of them gave a blue color with molybdic acid in ammoniacal solution. Portions of all three substances were heated at 135° *in vacuo* over sulfuric acid. The 4-imidopseudouric acid came to constant weight after 40 hours of heating; the two samples of 2-imidopseudouric acid after 8 hours.

4-Imidopseudouric acid from the sulfate of the 4,5-diamino base.

0.1024 gm. substance gave 0.1112 gm. CO_2 and 0.0408 gm. H_2O.
a. 0.0750 " " required 18.60 cc. 0.1 N HCl.
b. 0.0755 " " " 18.65 " 0.1 " "
0.3969 " " lost 0.0341 gm. weight when heated.
Dehydrated material.
a. 0.0736 gm. substance required 19.75 cc. 0.1 N HCl.
b. 0.0772 " " " 20.65 " 0.1 " "

2-Imidopseudouric acid from the sulfate of the 2,5-diamino base.

0.1038 gm. substance gave 0.1126 gm. CO_2 and 0.0416 gm. H_2O.
a. 0.0749 " " required 18.30 cc. 0.1 N HCl.
b. 0.0755 " " " 18.55 " 0.1 " "
0.5191 " " lost 0.0466 gm. weight when heated.
Dehydrated material.
a. 0.0791 gm. substance required 21.20 cc. 0.1 N HCl.
b. 0.0980 " " " 26.40 " 0.1 " "

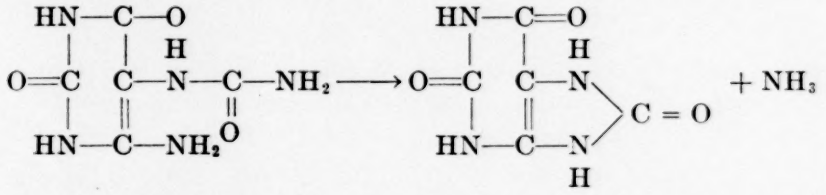
2-Imidopseudouric acid from the sulfate of divicine.

0.1008 gm. substance gave 0.1082 gm. CO_2 and 0.0413 gm. H_2O.
a. 0.0523 " " required 12.85 cc. 0.1 N HCl.
b. 0.0503 " " " 12.30 " 0.1 " "
0.1004 " " lost 0.0088 gm. weight when heated.
Dehydrated material.
a. 0.0455 gm. substance required 12.25 cc. 0.1 N HCl.
b. 0.0461 " " " 12.35 " 0.1 " "

	Calculated for $C_5H_7N_5O_2.H_2O$:	Found:		
		Substance from sulfate of 4, 5-diamino base.	Substance from sulfate of 2, 5-diamino base.	Substance from sulfate of divicine.
C.....	29.54	29.62	29.59	29.39
H.....	4.47	4.45	4.48	4.60
N.....	34.49	a.34.74 b.34.60	a.34.23 b.34.42	a.34.42 b.34.26
H_2O.....	8.87	8.59	8.98	8.8
	For $C_5H_7N_5O_2$:			
N.....	37.84	a.37.59 b.37.48	a.37.55 b.37.74	a.37.71 b.37.53

Conversion of the Pseudouric Acids into Uric Acids.—It was originally intended to convert the 4-imidopseudouric acid and the 2-imidopseudouric acid from both the synthetic and the natural pyrimidines into uric acid and 2-imidouric acid, respectively. But the very poor yields of 2-imidopseudouric acid obtained from the sulfate of divicine prevented the carrying out of the latter half of this plan.

Uric Acid from 4-Imidopseudouric Acid.—This conversion was carried out by a method similar to that used by Fischer[10] in converting 2-imidopseudouric acid into 2-imidouric acid, the only difference being that a molecule of ammonia instead of a molecule of water was eliminated.



1 gm. of 4-imidopseudouric acid was sealed in a bomb tube with 3 cc. of concentrated hydrochloric acid, and heated for 2 hours at 120°. The tube was constantly shaken during this period. When cooled and opened, its contents consisted of a brownish granular mass insoluble in water. This substance was dissolved in dilute sodium hydroxide, the solution boiled with bone-black, filtered, and poured into an excess of hot, dilute hydrochloric acid. There was an immediate crystalline precipitate. This was cooled, filtered out, and dried. The yield was 0.6 gm. The substance was boiled for 20 minutes with 100 cc. of 5 per cent hydrochloric acid, and filtered out. Then the purification process was repeated once more in an exactly similar fashion. The final material was perfectly crystalline and almost pure white. It was filtered out, washed with water, and dried *in vacuo* over sulfuric acid at 106°. The substance gave the murexide test with nitric acid, and formed with silver nitrate a white complex insoluble in ammonia.

0.0995 gm. substance gave 0.1305 gm. CO_2 and 0.0233 gm. H_2O.
0.0754 " " required 18.00 cc. 0.1 N HCl.
0.0766 " " " 18.25 " 0.1 " "

	Calculated for $C_5H_4N_4O_3$:	Found:
C	35.69	35.77
H	2.40	2.62
N	33.34	33.45 33.38

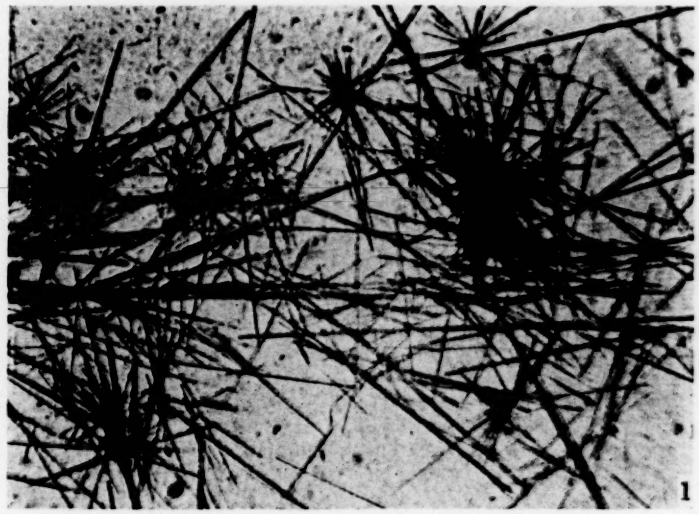
These data are considered to prove that the substance is uric acid.

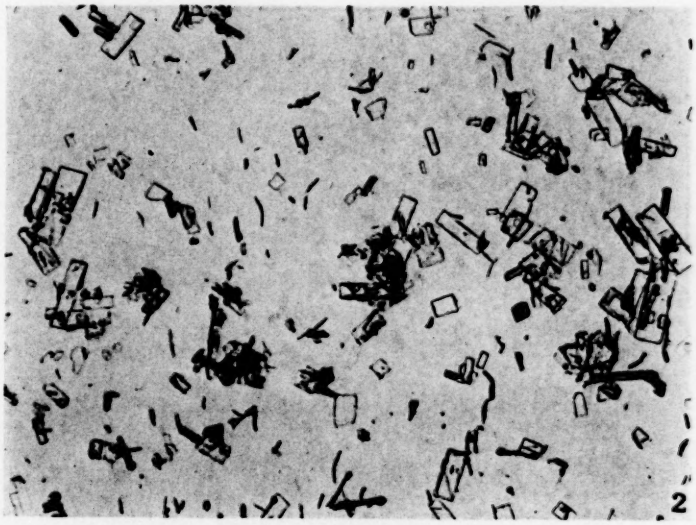
EXPLANATION OF PLATE 6.

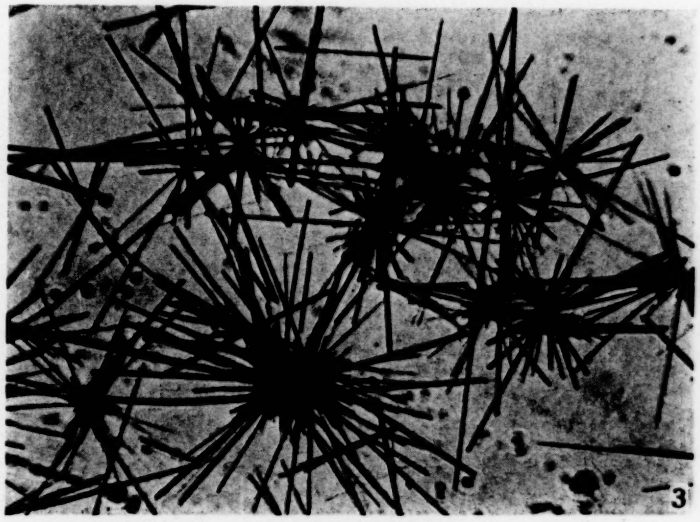
FIG. 1. 2-Imidopseudouric acid from the sulfate of 2,5-diamino-4,6-dioxypyrimidine.

Fig. 2. 4-Imidopseudouric acid from the sulfate of 4,5-diamino-2,6-dioxypyrimidine.

FIG. 3. 2-Imidopseudouric acid from the sulfate of divicine.







(Levene and Senior : Vicine and Divicine.)

THE PREPARATION OF GUANIDINE SULFATE.

By P. A. LEVENE and JAMES K. SENIOR.

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(Received for publication, May 31, 1916.)

In the course of work on divicine[1] it was necessary to prepare large quantities of a guanidine salt from which free guanidine could easily be obtained. The sulfate and carbonate suggested themselves, since both would give the free base on being treated with barium hydroxide. As no convenient method for preparing these salts could be found in the literature, it was necessary to devise one. This was successfully accomplished, and the preparation is here described in the hope that it may be of use to others.

The starting material was dicyandiamide

$$H_2N-\overset{\overset{NH}{\|}}{C}-N-C\equiv N \quad H$$

which was easily prepared from crude commercial calcium cyanamide by the method of Söll and Stutzer.[2] The yield was about 155 gm. per kg. of crude material.

0.0523 gm. substance required 24.75 cc. 0.1 N HCl.
0.0515 " " " 24.30 " 0.1 " "

	Calculated for $C_2H_4N_4$:	Found:	
N.....	66.65	66.30	66.11

100 gm. of dicyandiamide were mixed in a 2 liter wide-mouthed round-bottomed flask with 200 cc. of 75 per cent sulfuric acid. After about 2 minutes the substance grew very hot, and there was a violent evolution of carbon dioxide which lasted for several

[1] Levene, P. A., and Senior, J. K., *J. Biol. Chem.*, 1916, xxv, 607.
[2] Söll, J., and Stutzer, A., *Ber. chem. Ges.*, 1910, xlii, 4533.

minutes. When this spontaneous reaction had nearly ceased, the flask was gently heated on a Babo funnel for 5 minutes, at a temperature sufficient to keep a good stream of gas coming off. The flask was then allowed to cool. At the moment when the contents began to solidify, 1,500 cc. of 95 per cent alcohol were added. The mixture was then cooled in the ice chest over night. The solid material (which consisted of almost pure ammonium sulfate) was filtered out and washed with 95 per cent alcohol. The filtrate and washings were combined and concentrated to a volume of about 300 cc. The liquid was then transferred to a large flask on the steam bath, and, while constantly stirred, was neutralized with barium carbonate. The solid barium salts were then filtered out. The liquid, which was usually a trifle alkaline, was just neutralized with dilute sulfuric acid, and concentrated to a volume of about 200 cc. It was then filtered and further concentrated to a thick oil. This oil was poured into 1 liter of 95 per cent alcohol. When stirred and scratched, it slowly solidified into a crystalline mass. The flask was cooled in the ice chest, the solid filtered out, washed with 95 per cent alcohol, and dried in the air bath at 100°. The yield was 125 gm.

a. 0.0501 gm. substance required 13.00 cc. 0.1 N HCl.
b. 0.0509 " " " 13.15 " 0.1 " "
a. 0.3008 " " gave 0.3162 gm. $BaSO_4$.
b. 0.3023 " " " 0.3175 " "

	Calculated for $(CN_3H_5)_2H_2SO_4$:	Found: a.	b.
N	38.88	36.35	36.20
H_2SO_4	45.36	44.17	44.13

The nature of the impurities present was never determined as the substance was sufficiently pure for the desired purposes, and was used without further refinement.

THE PITUITARY GLAND. ITS EFFECT ON GROWTH AND FISSION OF PLANARIAN WORMS.

By ROSALIND WULZEN.

(*From the Rudolph Spreckels Physiological Laboratory of the University of California, Berkeley.*)

(Received for publication, May 2, 1916.)

The recorded effects of the pituitary gland on growth and reproduction are so varied that further investigation of the subject seems to be desirable.

Many[1] have found an inhibition in growth. On the other hand, Robertson and Burnett[2] report acceleration in growth of carcinomata, and Robertson[3] gives convincing experiments pointing to an early retardation and a later acceleration of growth in white rats. Goetsch[4] also reports increase in growth with rats under pituitary feeding. Some have observed in addition a sexual effect. Cushing[1] points out the wide occurrence of sexual disturbances as an accompaniment of pituitary disorders in clinical cases. He and also Aschner[5] find that the operative removal of the pituitary in young animals leads to sexual infantilism. Behrenroth[6] and Goetsch[4] have observed precocious sexual development accompanying treatment with the pituitary gland. Clark[7] gets increased egg production of hens on feeding pituitary. Pearl,[1] on the other hand, finds that in pullets pituitary substance does not bring about an earlier activation of the ovary than occurs in controls.

A local planarian worm *Planaria maculata* was selected as a good subject for experimentation because it will live and thrive

[1] Cerletti, U., *Arch. ital. biol.*, 1907, xlvii, 123. Sandri, O., *ibid.*, 1909, li, 337. Goetsch, E., and Cushing, H., cited by Cushing, The Pituitary Body and Its Disorders, Philadelphia, 1912, 11. Aldrich, T. B., *Am. J. Physiol.*, 1912, xxx, 352; 1912–13, xxxi, 94. Wulzen, R., *ibid.*, 1914, xxxiv, 127. Pearl, R., *J. Biol. Chem.*, 1916, xxiv, 123.

[2] Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1915, xxi, 280.

[3] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 385.

[4] Goetsch, E., *Bull. Johns Hopkins Hosp.*, 1916, xxvii, 29.

[5] Aschner, B., *Arch. ges. Physiol.*, 1912, cxlvi, 1.

[6] Behrenroth, E., *Deutsch. Arch. klin. Med.*, 1914, cxiii, 393.

[7] Clark, L. N., *J. Biol. Chem.*, 1915, xxii, 485.

upon a meat diet exclusively, and because its small size makes possible the administration of a constant excess of food. Also the readiness with which it reproduces in the laboratory makes easy the study of the effects of diet upon this function. Child,[8] who has made exhaustive studies of the process of fission in *Planaria*, holds that the formation of a new worm from the piece resulting from fission "is as truly a reproduction as the process of the formation of a planarian from the egg." Hence changes in the rate of fission would indicate changes in the reproductive function of the worms just as surely as would changes in the rate of production of offspring by a mammal.

After being collected the worms were fasted in the laboratory for at least 2 weeks. In the second series very small worms were used. These were obtained by allowing tips cut from the posterior ends of fasting worms to regenerate. They then had a length of 3 mm. or less, and according to Child[8] must be considered as very young worms. The animals were kept in finger bowls and the water upon them was often changed. They were fed almost without exception four times a week and were always given more than they could consume. The food was left before them over night. Throughout the experiment they gathered upon all varieties of meat given them and ate greedily. Data were collected upon the occurrence of fission, changes in length, and the final weight. The worms were measured in a flat-bottomed dish under which was a rule that could be moved. They were stirred to stimulate them to move with the maximum extension, and as they passed over the lines of the ruler they were measured. While this method is not exact because of the varying tonus of the worms, the measurements obtained indicate the size with an accuracy amply sufficient for the purposes of the experiment.

EXPERIMENTAL.

In the first series the worms were divided into three lots: those below 5 mm., those between 5 and 10 mm., and the largest ones, which measured a little below or above 10 mm. Each lot was again divided into two equal portions, one of which was fed upon pars glandularis of ox pituitary and the other upon liver. The

[8] Child, C. M., *Arch. Entwcklngsmechn. Organ.*, 1913, xxxv, 598.

worms soon began to divide so that measurements of the original individuals became useless. It seemed best to consider all the new worms as still being a part of the worm from which they sprang. Hence to get the average size of a group, all the pieces of planarian in the dish were measured and the sum was divided by the original number of worms in that group. In considering the length one must bear in mind that the new worms produced by fission elongate by readjustment of substance to take on the typical form. This would tend to increase the length of planarian substance of worms which had divided often over those which had not. On the other hand, newly divided worms do not eat for some time. Those which have not divided are eating and growing in this interval. The two factors tend to counterbalance each other. Feeding began in October, and the dates given as final were taken in March. At the conclusion the worms in each dish were weighed after being dried with filter paper. The results are stated in Table I.

TABLE I.

Series 1.

Group.	Food.	Original No.	Final No.	Average increase.	Ratio of increase.	Original total length.	Final total length.	Average increase.	Ratio of increase.	Final total weight.	Final average weight.	Ratio of final weight.
						mm.	*mm.*	*mm.*		*gm.*	*gm.*	
A. Small.	Liver....	27	40	0.48	1	141	356	7.9	1	0.159	0.00588	1
	Pituitary	27	73	1.70	3.54	116	514	14.7	1.86	0.197	0.00729	1.23
B. Medium.	Liver....	32	85	1.65	1	214.5	918.5	22	1	0.496	0.0155	1
	Pituitary	33	132	3.00	1.81	254	951.5	21.1	0.95	0.395	0.0119	0.76
C. Large.	Liver....	22	66	2.00	1	251	788	24.4	1	0.285	0.0129	1
	Pituitary	19	78	3.10	1.55	186	683.5	26.1	1.06	0.274	0.0144	1.11

It will be noted that the rate of fission in *Planaria* fed upon pituitary is in all cases decidedly larger than in the controls. The worms which were smallest in the beginning show the greatest effect, their rate of division being 3.54 times as great as that of the controls. The medium worms show an increase 1.81 times as great, and the largest worms 1.55 times as great as those fed

on liver. In length the medium and large pituitary worms developed almost equally with the controls. But the small worms increased 1.86 times as much in length as those fed on liver. Though the weights were not taken at the beginning of the experiment they must have been very nearly equal for the two divisions of each group, because the worms were equal in measurement and were in a starving condition at the time.

Here again we see a real difference in weight in favor of those fed upon pituitary in the case of the smallest worms. With the medium worms the controls are heavier. It should be remembered, however, that the worms fed upon liver have the appearance of being fatter than those eating pituitary. It seems that the liver diet leads to a storage of reserve material. If this is true, the weight of the liver-fed worms is greater than it should be, and any excess in favor of pituitary feeding, as shown in Group A, is more significant. During the time of this experiment the larger worms do not show a definite difference from the controls in length or weight. Judging, however, from the results obtained with small worms, these larger worms ought also to exceed the controls, were the time sufficient for the new individuals, produced by fission and subjected from inception to pituitary influence, to become adults; Group B is being continued with this idea in mind.

A second series was undertaken to demonstrate, if possible, differences in the effect of the various portions of the pituitary. Because of the uncertainty arising from fission in the first series and because of the greater effect noted in the younger individuals, very small worms were chosen for this experiment. They were almost all less than 4 mm. in length. Six groups nearly equal in size and number were formed. Group 1 was fed on pars glandularis. A portion well away from the cleft and in the center of the gland was used; this includes the cone of sinusoid vessels which pass from hilus to stem and contain large amounts of secretion. Group 2 was fed on cone substance. The cone is a structure appearing upon the pars intermedia in the ox pituitary.[9] In obtaining this food it was so cut that no pars intermedia was included with it. A large number of glands yield only a small

[9] Wulzen, R., *Anat. Rec.*, 1914, viii, 403.

amount of this substance. Group 3 was fed on pars intermedia cut carefully away in strips from pars posterior. Group 4 was given a portion of pars posterior from which pars intermedia was cut away. Group 5 had liver and Group 6 white matter of the brain. The feeding was continued until fission began to interfere with the results. After this no more food was given, but the dishes were allowed to stand so that the number of fissions might be observed. The data appear in Table II.

TABLE II.

Series 2.

Group.	1. Pars glandularis.		2. Cone.		3. Pars intermedia.		4. Pars posterior.		5. Liver.		6. White matter, brain.	
Date.	No.	Average length.	No.	Average length.	No.	Average length.	No.	Average length.	No.	Average length.	No.	Average length.
		mm.		*mm.*		*mm.*		*mm.*		*mm.*		*mm.*
Nov. 12	48	3.1	49	2.9	50	2.8	47	3.0	46	3.0	47	3.0
" 22	48	3.7	42	3.6	48	3.8	47	4.0	44	3.5	47	3.5
Dec. 2	48	4.36	41	4.26	43	4.27	47	4.07	44	3.67	38	3.2
" 15	48	5.75	40	5.23	39	5.55	41	5.21	44	4.72	35	3.35
" 29	48	7.24	40	5.81	39	6.96	41	6.43	42	5.88	34	3.30
Jan. 19	48	7.82	38	6.44	39	7.11	41	6.56	42	6.21	25	2.96
Feb. 9	46	9.60	37	7.44	37	8.98	39	7.83	40	7.41	19	3.02
Total weight on Feb. 9, gm.	0.174		0.077		0.124		0.109		0.116			
Average weight, gm.	0.00378		0.00208		0.00335		0.00279		0.00290			
No. of new individuals on Mar. 13.	32		11		17		32		3			

The decreasing number of individuals during the experiment is due to the occasional loss of a worm in washing the dishes. But it expresses more than this in Group 6 where a harmful effect of the diet is certainly shown. In length and weight pars posterior, cone, and liver gave results so nearly equal that these portions of the pituitary may be called neutral as regards the growth of *Planaria*. It should be noted, however, that those fed on cone fall below in weight. The two remaining portions of the pituitary gave positive results. The worms fed upon pars

glandularis show an undoubted excess both in size and weight over the liver-fed controls. Those receiving pars intermedia have also increased in size and weight though not quite so much. The brain-fed worms which were intended as controls did not grow at all after the first 2 weeks. The varying values for their length are explained by the death of a considerable number of individuals. The meaning of this inhibition is unknown and will be investigated further. A striking result is to be noted in the number of new individuals produced by fission. No matter what the portion of pituitary fed, the rate of fission was greatly increased thereby. Pars glandularis and pars posterior gave a rate more than ten times as great as liver, while pars intermedia showed a rate almost six times, and cone substance one almost four times as great as liver.

DISCUSSION.

These experiments indicate positive effects on growth and reproduction when pituitary is fed to planarian worms. But they indicate further a separation in these effects. In Series 1, increased growth is shown in a pronounced way only by those worms which had received the diet from a very early period, while the effect on reproduction is marked no matter what the size (that is, age) of the worms when feeding was begun.

This result is one which reproduces a series of experiments of my own upon the growth of chicks. These experiments have not been previously reported by me as they needed the confirmatory evidence from other sources which now exists. In the first experiment I succeeded in raising two cocks from baby chicks to the age of 9 months. One received large amounts of fresh pars glandularis of ox pituitary, about $\frac{1}{100}$ of the body weight, almost every day. The other had corresponding amounts of liver. During the early growth period the pituitary-fed chick showed inhibition of growth as did the chicks in my recorded experiments[1] and also those of Pearl.[1] However, when they became 4 months old, the control stopped growing rapidly while the pituitary cock continued its rapid growth and in 3 weeks had become larger and heavier than the control. This growth continued until the pituitary cock surpassed the control considerably. Its weight was then 1,882 gm. while that of the control was 1,597 gm. Its foot measured 206 mm. against 157 mm. of the control. This foot measurement exceeded that of any of the twelve cocks raised subsequently by me, presently to be reported. The resemblance between this result and that reported by Robertson[3] is remarkably close. Furthermore, the pituitary

cock began to crow at the age of 2 months, 2 weeks before the control, and throughout its life it crowed much oftener than did the control. The feathering of the pituitary cock was much handsomer, whiter, and silkier, and the feathers were longer and more downy than the control's.

In another series, twelve cocks, of the same breed as the above, were divided into two equal groups. Each cock in one group received about 15 gm. of fresh pars glandularis almost every day, while the others received the same amount of liver. They were 4 months old at the start and had not attained their full growth. The feeding continued for 3 months and at the end of that time no distinction could be made between the two groups as to size. But the crowing showed the same difference as before. It was counted for certain intervals of time in the middle of the day. During the major part of the experiment there were always many more crows given by the pituitary cocks than by the controls. Sometimes these would crow twenty times before the others crowed once. At the conclusion of the experiment they were crowing more equally, but it seeemed as though all crowed more continually than do ordinary cocks. If the two lots could have been separated, the difference in number of crows would undoubtedly have been greater still and would probably have persisted to the end. The vigorous crowers always started the others going and kept them at it.

Thus it seems with both fowl and *Planaria* that the feeding of pituitary must be begun in extreme youth and continued over prolonged periods in order that the effect on growth may appear. On the other hand, its effect upon reproduction in *Planaria* and the secondary sexual character of crowing in fowls is clearly defined even when feeding is begun with individuals well along toward the adult condition. The failure of pituitary feeding begun in the adolescent period to accelerate the growth of fowl might well be due to the advanced stage of ossification in the bones, but this cannot be true of the flat worms where there is no known anatomical difference between very large and very small individuals.

This perhaps indicates a distinction between the substance which produces growth and that which affects reproduction. The idea that some difference exists is strengthened by the second series of experiments with *Planaria*. These show that growth is accelerated by pars glandularis and pars intermedia but not at all by pars posterior, whereas rate of fission is increased by pars glandularis and pars posterior equally. It seems as though the substance which acts upon reproduction is stored everywhere in the gland and it is therefore able to show its effect when com-

paratively small quantities are eaten, but the growth substance, present in pars glandularis and pars intermedia, is quickly lost from the gland. Therefore to show its effect feeding must begin when tissues are young and very sensitive and must be continued over long periods of time.

This work gives a little indication as to the path of exit of secretion from the pituitary gland. From the evidence gathered by them, Herring,[10] Cushing and Goetsch,[1] and Cow[11] conclude that the substance secreted by pars intermedia leaves the gland through pars posterior and passes into the ventricle of the brain. But my studies with the pituitary of the ox, both gross and histological, have given me the impression that much more secretion must pass from the gland through the large blood vessels than can enter the brain. Thaon[12] has described and pictured blood vessels of pars glandularis gorged with secretion. I have repeated Thaon's work and have seen sinus after sinus packed with secretion of pars glandularis. The colloid substance present so often in the cleft of the ox pituitary has every appearance of being a product of pars intermedia. Blood vessels in the cleft region of pars glandularis, especially about the portion which is in contact with the cone, are very numerous and close to the surface and must offer admission to any substance present in the cleft. The path from pars intermedia to the blood sinuses of pars glandularis appears to offer much less resistance to the passage of secretion than that by way of pars posterior If pars intermedia secretion passes largely through pars posterior, then pars posterior should incite growth as do pars glandularis and pars intermedia. But in this investigation upon *Planaria* it does not, nor has any one else reported such a result.

If we grant that pars intermedia sends its secretion mainly into pars glandularis, the question arises why pars posterior has so large an effect upon fission. If a guess might be made it would perhaps be this: The fresh secretion of the pituitary is produced by pars glandularis and pars intermedia, and most of it is carried quickly away by the blood vessels. This is the substance which is active in producing growth as well as in effecting

[10] Herring, P. T., *Quart. J. Exp. Physiol.*, 1908, i, 121.
[11] Cow, D., *J. Physiol.*, 1914–15, xlix, 375.
[12] Thaon, P., Thèse de Paris, 1907.

reproduction. All the secretion does not find its way into the blood vessels, however; some works out slowly through the posterior lobe. Moreover, there are in all portions of the gland blind alleys where masses of the secretion are brought to a standstill. As it lies in these cysts the character of the secretion changes. It loses its power over growth but can still affect sexual function. Thus pars posterior which has usually stored away much secretion can effect reproduction. It does not effect growth because no large amounts of fresh secretion are present in it.

I have attained positive effects upon fission with a diet of pars posterior while others have found nothing resulting but weakness and emaciation. This may be because *Planaria* are not injured by this substance so that in them it can produce its physiological effect. Apparently the higher animals are too severely harmed by this diet.

CONCLUSIONS.

1. The rate of fission in planarian worms is increased by a diet of pituitary substance, no matter what portion of the gland is used or what the age of the worms involved.

2. The growth of planarian worms is accelerated by a diet of pars glandularis and pars intermedia, provided that the worms are very small when the diet is begun.

3. There is indication of a distinction between the substance which produces fission and that which produces growth.

4. There is indication that the growth-producing substances of pars glandularis and pars intermedia leave the gland by way of the blood vessels of pars glandularis.

EXPERIMENTAL STUDIES ON GROWTH.

V. THE INFLUENCE OF CHOLESTEROL UPON THE GROWTH OF THE WHITE MOUSE.

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(Received for publication, May 25, 1916.)

Previous Knowledge Concerning the Influence of Cholesterol upon Growth.

Previous investigations in this laboratory[1] have shown that cholesterol, when administered subcutaneously in localities either near to or remote from the primary tumor, causes a very marked acceleration of the growth of Flexner-Jobling carcinoma in rats and also decidedly enhances the tendency of the primary tumor to give rise to metastases. The effect of cholesterol upon the growth of carcinomata is therefore analogous to that exerted by emulsions of the anterior lobe of the pituitary body[2] and by the growth-controlling principle, tethelin, which has been isolated from the anterior lobe of the pituitary body.[3]

It has also been shown that cholesterol, emulsified in hay infusion, causes a marked acceleration of the rate of multiplication of paramecia.[4]

The decided effects exerted by cholesterol upon the growth of carcinomata and the division rate of paramecia suggested the possibility that cholesterol, if added to the diet in unusual quantities, might have an appreciable effect upon the growth of mammals. The experiments which are about to be described were accordingly undertaken.

[1] Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1913, xvii, 344. Burnett, T. C., *Proc. Soc. Exp. Biol. and Med.*, 1913, xi, 42; 1914, xii, 33.

[2] Robertson and Burnett, *J. Exp. Med.*, 1915, xxi, 280.

[3] Robertson, T. B., *J. Biol. Chem.*, 1916, xxiv, 397, 409. Robertson and Burnett, *J. Exp. Med.*, 1916, xxiii, 631.

[4] Browder, A., *Univ. California Publications, Physiology*, 1915, v, 1.

Description of the Experiments. Mode of Administering the Cholesterol. Dose Administered.

Average healthy young mice, about 5 weeks of age, were taken at random from our stock, transferred to the cages described in the first article of this series,[5] and immediately, and thereafter until the age of 60 weeks, fed with cholesterol. Thirty-four males and thirty-five females were employed for this purpose. In every respect other than in the administration of the cholesterol these animals were handled and fed exactly as were the normal animals.[6] Their cages were placed in the same room as and not far removed from those containing the normal animals. They were only about 3 months younger than the normal animals and hence their life and growth were contemporary with the greater part of the life and growth of the normals. The cholesterol-fed animals and the normal animals were always weighed in the middle of the afternoon. Hence no possible factor other than the administration of cholesterol could account for the observed deviations of the growth of these animals from the normal.

The cholesterol employed was Merck's. The mixed whites and yolks of eggs were beaten and strained and the cholesterol, in the proportion of 1 gm. of cholesterol to 20 cc. of egg mixture, was rubbed up with the egg until it formed a smooth creamy emulsion. 5 cc. of this emulsion were placed in each compartment containing six mice, every day in the week except Sunday. The emulsion was eagerly eaten by the mice so that all of the cholesterol administered was consumed. The daily dose of cholesterol per mouse, added to that already present in the food, was therefore about 42 mg. The proportionate dose decreased with age, for it was not increased as the animals grew heavier. It was also somewhat larger for the females than for the males, since the females are somewhat less heavy than the males.

Results.—The weights of the cholesterol-fed and the normal animals are compared in Tables I and II and graphically illustrated in Figs. 1 and 2, the undotted curves representing the normal growth of white mice during the experimental period,

[5] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347.
[6] Robertson, *J. Biol. Chem.*, 1916, xxiv, 363.

the dotted curves the growth of the animals which received cholesterol. The dots represent the positions of the actual observations.

The effect of the administration of cholesterol is qualitatively the same in both sexes and quantitatively similar. Between the 5th and 10th weeks growth is very markedly retarded, so much so that at 10 weeks of age the cholesterol males lag 3.78 gm. and the females 3.84 gm. behind the normal animals. Between the 10th and 60th weeks, however, the growth of the cholesterol-fed animals is very decidedly accelerated, so that although they fail to catch up to the normals, yet at 20 weeks of age the cholesterol males lag only 2.73 gm. and the females 2.48 gm. behind the normals, the males having made up 1 gm. and the females 1.4 gm. of the defect due to the initial retardation. The subsequent

TABLE I.

Cholesterol-Fed Male Mice.

Age.	Weight.		No. weighed. (Cholesterol-fed.)	Age.	Weight.		No. weighed. (Cholesterol-fed.)
	Normal.	Cholesterol-fed.			Normal.	Cholesterol-fed.	
wks.	*gm.*	*gm.*		*wks.*	*gm.*	*gm.*	
4	12.38		2	25	27.05	24.24	29
5	12.45	12.84	22	26	26.94	24.48	29
6	15.58	14.06	33	27	26.55	24.16	29
7	18.08	15.85	34	28	27.19	25.00	29
8	19.36	16.88	34	29	27.08	24.59	29
9	20.63	16.79	34	30	27.23	24.77	28
10	21.19	17.41	34	32	27.61	25.34	34
11	21.81	17.85	34	34	27.71	25.62	34
12	22.65	18.96	34	36	27.95	25.66	34
13	23.31	20.46	34	38	28.20	26.18	33
14	23.96	21.37	34	40	28.27	26.06	27
15	24.28	21.88	34	42	28.28	26.19	32
16	24.75	22.03	34	44	28.62	26.27	32
17	25.21	22.20	32	46	28.55	27.00	32
18	25.61	23.10	34	48	28.22	26.84	32
19	25.81	22.55	32	50	28.88	26.83	32
20	26.10	23.37	34	52	28.68	26.78	32
21	26.28	23.47	30	54	28.67	27.50	32
22	26.06	24.19	34	56	29.32	26.98	32
23	26.34	24.02	30	58	29.10	26.78	32
24	26.82	24.43	29	60	29.08	26.88	32

TABLE II.

Cholesterol-Fed Female Mice.

Age.	Weight.		No. weighed. (Cholesterol-fed.)	Age.	Weight.		No. weighed. (Cholesterol-fed.)
	Normal.	Cholesterol-fed.			Normal.	Cholesterol-fed.	
wks.	*gm.*	*gm.*		*wks.*	*gm.*	*gm.*	
4	10.39		2	25	23.79	20.94	33
5	11.81	13.14	14	26	24.04	21.24	33
6	14.12	13.10	35	27	24.00	21.32	33
7	16.77	13.90	35	28	23.58	21.56	33
8	17.99	14.90	35	29	23.84	21.62	33
9	18.78	15.10	35	30	23.92	22.07	34
10	19.38	15.54	35	32	24.18	22.08	33
11	20.04	16.06	35	34	24.18	22.28	32
12	20.31	17.10	35	36	24.65	22.41	32
13	21.04	17.73	35	38	24.80	22.89	32
14	21.21	18.46	35	40	25.03	22.88	32
15	21.78	18.77	35	42	25.07	22.90	31
16	22.14	19.21	35	44	25.52	23.30	30
17	22.29	19.30	33	46	25.68	24.14	29
18	22.22	19.66	34	48	25.45	23.86	29
19	22.60	19.65	33	50	25.50	23.86	28
20	22.60	20.12	34	52	25.76	24.06	27
21	23.06	20.24	33	54	25.78	24.61	27
22	23.32	20.35	34	56	26.00	24.30	27
23	23.51	20.48	33	58	26.26	24.13	26
24	23.68	20.80	33	60	26.12	24.35	26

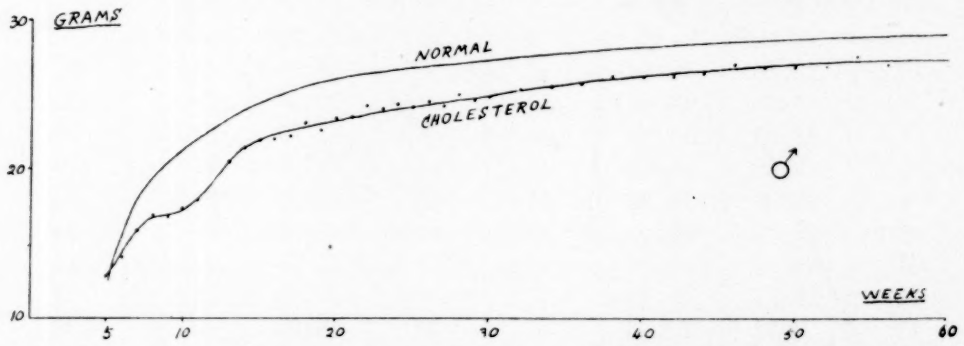

FIG. 1. Comparison of the growth curves of normal and of cholesterol-fed males.

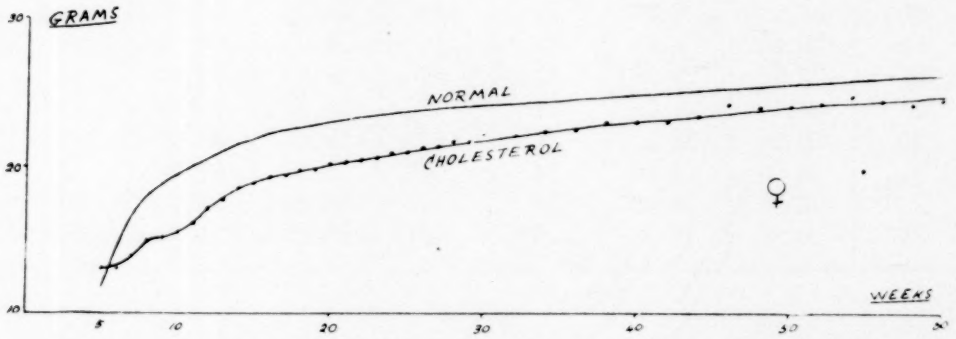

FIG. 2. Comparison of the growth curves of normal and of cholesterol-fed females.

acceleration of growth is slight but consistent, so that the growth curves of the cholesterol-fed and normal animals from the 20th to the 60th weeks slowly but continuously approach one another. At 60 weeks of age the cholesterol-fed males only lag 2.20 gm. behind the normal males, having made up between the 20th and 60th weeks an additional 0.5 gm. of the defect due to the early retardation of growth, while the females lag 1.77 gm. behind the normals, having made up in the same time an additional 0.7 gm. of the defect. The initial retardation and subsequent acceleration of growth are so marked as to cause complete distortion of the growth curve, giving the appearance either of great prolongation and enlargement of the second growth cycle, as in the males, or of a new growth cycle interpolated between the second and third cycles in the normal curve, as in the females, the third growth cycle in both sexes being accelerated and curtailed. On comparing the growth curves of the cholesterol-fed animals with the growth curves of tethelin-fed animals[7] the close analogy between the two sets of growth curves cannot fail to be observed. It is evident that in their effect upon the third growth cycle of mice, as in their effect upon the growth of carcinomata, tethelin and cholesterol are analogous. In their potency these substances differ very greatly, however, for the effect upon the growth of mice which is brought about by the administration of a daily dose of 40 mg. of cholesterol is achieved by the daily administration of one-tenth of that amount of tethelin.

On comparing the variabilities of cholesterol-fed and normal animals (Tables III and IV and Figs. 3 and 4) a striking difference between the actions of cholesterol and of tethelin upon growth is revealed, for whereas tethelin causes a marked diminution in the variability of the animals to which it is fed, cholesterol gives rise to an equally marked increase. This is very clearly displayed in the case of the females (Fig. 4), and that the same effect was also exerted upon the variability of the males (Fig. 3) will be evident on observing that by chance the males selected for the experiment were initially (*i.e.*, at 5 weeks) much less variable than the average normals, the variability at 5 weeks of the males selected for the experiment being only 18.1 per cent

[7] Robertson, *J. Biol. Chem.*, 1916, xxiv, 397.

while the average variability of normal males at that age is 24.6 per cent. Yet between the 8th and 12th weeks the variability of the cholesterol-fed males exceeded that of the normals, and subsequent to 12 weeks the variability of the cholesterol-fed males remained very nearly equal to that of the normals, indicating that had the initial variability of the males selected for the experiment been normal, their variability after feeding them with cholesterol would have considerably exceeded that of the normals.

TABLE III.

Cholesterol-Fed Male Mice.

Age.	Variability.		Age.	Variability.	
	Normal.	Cholesterol-fed.		Normal.	Cholesterol-fed.
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cent*
5	24.6	18.1	26	10.8	10.5
6	22.0	19.3	27	11.3	9.2
7	16.9	16.2	28	10.9	10.4
8	15.9	15.5	29	10.9	9.1
9	16.5	17.9	30	9.5	11.2
10	16.7	18.4	32	9.6	11.1
11	13.3	18.2	34	10.5	11.3
12	13.7	16.1	36	10.1	11.8
13	14.2	13.5	38	10.5	9.9
14	14.1	12.4	40	10.4	11.8
15	11.9	13.3	42	11.2	11.5
16	12.7	12.9	44	12.0	10.5
17	12.4	11.0	46	11.7	10.4
18	12.1	11.5	48	10.8	9.4
19	12.1	9.9	50	11.1	9.3
20	10.8	11.0	52	10.7	10.5
21	11.2	9.1	54	12.0	9.9
22	9.0	10.5	56	12.0	9.8
23	9.8	9.4	58	10.9	11.3
24	10.1	10.3	60	11.8	10.8
25	11.0	8.9			

TABLE IV.

Cholesterol-Fed Female Mice.

Age.	Variability.		Age.	Variability.	
	Normal.	Cholesterol-fed.		Normal.	Cholesterol-fed.
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cent*
5	19.4	21.5	26	11.6	14.7
6	18.9	15.6	27	11.8	14.8
7	15.0	16.5	28	10.3	15.7
8	13.9	15.4	29	12.2	15.2
9	13.4	17.8	30	12.4	16.6
10	14.3	15.2	32	11.5	17.3
11	12.6	18.1	34	12.2	18.4
12	12.6	16.1	36	11.2	19.9
13	13.0	16.9	38	12.3	20.4
14	13.2	14.7	40	11.9	21.0
15	13.7	13.7	42	13.1	22.8
16	12.6	13.3	44	15.3	23.1
17	11.9	12.8	46	14.6	23.0
18	11.9	12.8	48	14.6	23.4
19	12.0	12.3	50	14.0	23.0
20	11.2	12.1	52	15.3	22.4
21	12.3	12.9	54	15.8	21.9
22	12.5	12.4	56	16.5	23.1
23	12.0	12.4	58	16.7	23.0
24	11.3	14.3	60	17.8	21.1
25	11.4	14.5			

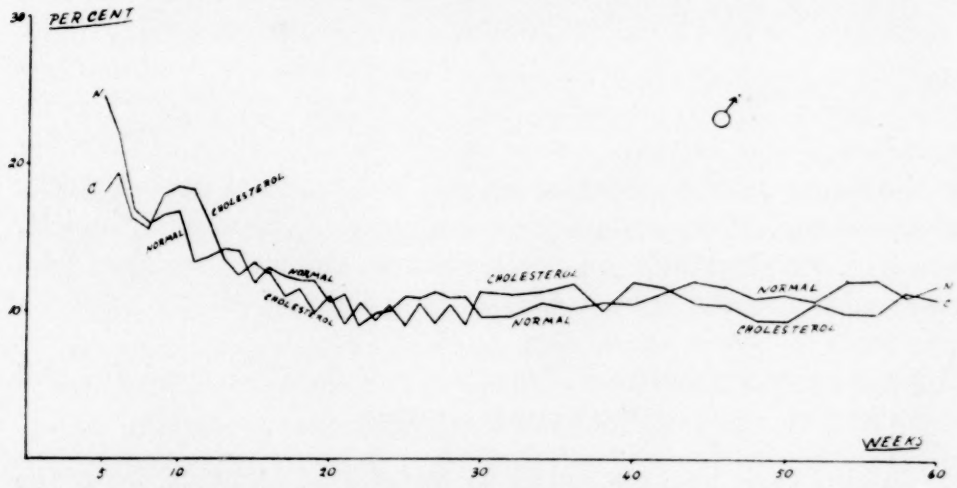

FIG. 3. Comparison of the variability curves of normal and of cholesterol-fed males.

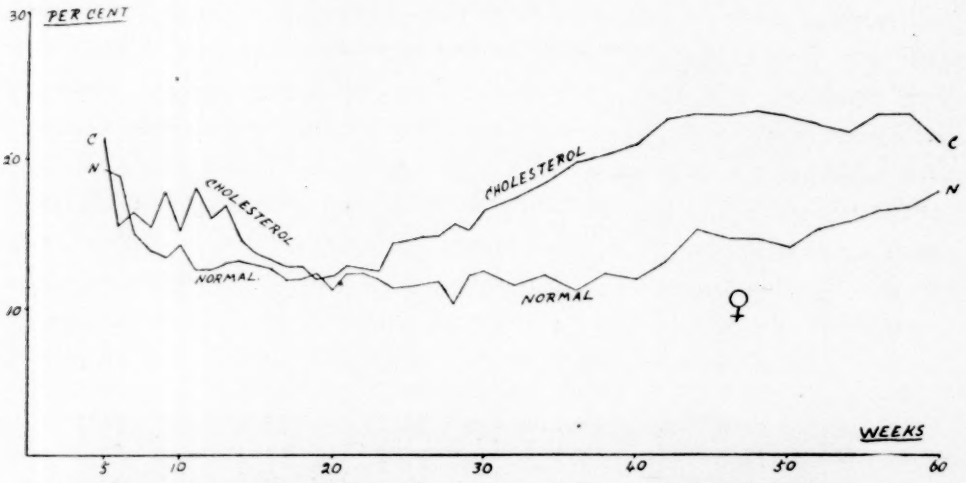

FIG. 4. Comparison of the variability curves of normal and of cholesterol-fed females.

The origin of the high variability of the cholesterol-fed animals lies in the fact that all of the animals do not display equal acceleration in the growth subsequent to 10 weeks of age. While the growth of some individuals is very greatly accelerated, producing, especially among the females, a group of very heavy animals, other individuals display little or no secondary acceleration of their growth. Since all of the animals are retarded in their growth during the first 5 weeks of the third growth cycle, this unequal acceleration in the latter part of the third growth cycle results finally in the production of a small group of very light animals, a larger group of animals of medium weight, and another small group of exceptionally heavy animals. In Fig. 5 female members of the extreme groups are compared. Both of the mice in the figure were 10 months old and had received since 5 weeks of age 40 mg. of cholesterol 6 days a week. The one on the left, however, weighed 14.5 gm. while that on the right weighed 44.5 gm. and 3 months later attained the weight of 48 gm.

Differences in the External Appearance of the Cholesterol-Fed and the Normal Animals.

Cholesterol is also analogous to tethelin in its effect upon the nutrition of the cutaneous tissues, for the coats of cholesterol-fed males are very noticeably superior in color and in smoothness

to those of normal males of the same age. This improvement of the coat, however, is not so striking as it is in the case of tethelin-fed animals. On the other hand the cholesterol-fed animals do not noticeably differ in the compactness of their build from normal animals of the same age, whereas tethelin-fed animals and animals fed upon the anterior lobe of the pituitary body, as I have pointed out in the articles alluded to above, differ very strikingly from normal animals in the superior compactness and heaviness of their build. The limbs and extremities of the tethelin- and pituitary-fed animals give the impression (although no

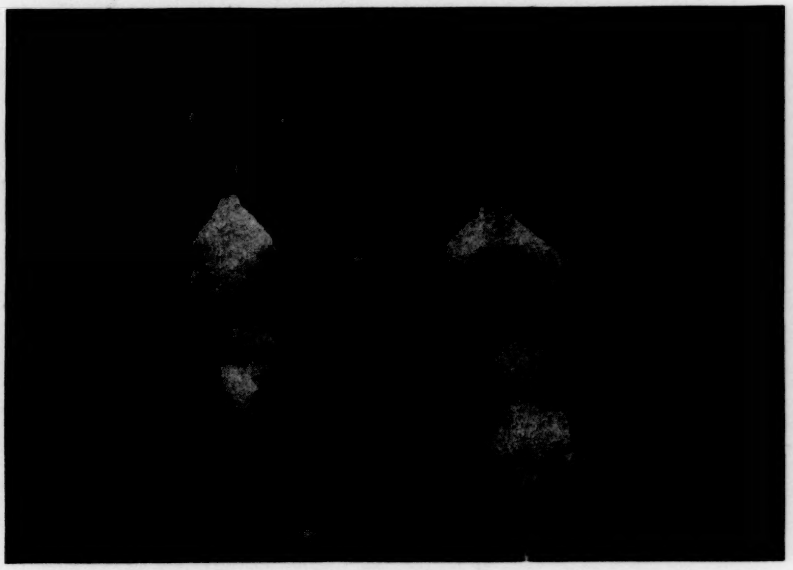

FIG. 5. Cholesterol-fed females, each 10 months of age, illustrating the high variability of cholesterol-fed animals.

actual measurements were made) of being thick and short and sturdy, while those of the cholesterol-fed animals are slender and delicate.

One very striking and invariable characteristic of cholesterol-fed males between the 5th and 10th weeks is the extreme prominence of the testicles. No actual measurements were made, but the impression was gained that while the body growth of cholesterol-fed males is strongly retarded, between the 5th and 10th weeks, the growth of the testicles is not similarly retarded, with the result that a striking disproportion between the size of the

animals and that of their testicles speedily becomes evident. Subsequent to the 10th week the cholesterol-fed males enter upon a period of rapid body growth and the disproportion in the size of the testicles disappears. This effect was observed in almost every male of the cholesterol-fed series, while it was not observed in any of the numerous experimental animals not receiving cholesterol.

Absence of Deleterious Effects upon the Health of the Animals Attributable to the Administration of Cholesterol.

It has been pointed out by Anitschkow,[8] Chalatow,[9] Bailey,[10] and others that the administration of cholesterol in daily doses of 0.2 to 0.8 gm. for periods of 50 to 100 days, or of equivalent amounts of cholesterol-containing foods such as brain or egg yolk, leads in rabbits to the formation of extensive deposits of anisotropic fat and ultimately to the formation of extensive lesions in the liver and other organs and to the formation of atheromata in the wall of the aorta, the formation of these lesions being accompanied by emaciation of the animal and ultimately leading to death. Anitschkow and Chalatow failed, however, to obtain similar lesions either in the liver or in the aorta in rats even after 5 months' feeding with a daily dose of one yolk of egg. Bailey[11] has similarly found that the daily administration of 0.1 to 0.5 gm. of cholesterol to guinea-pigs, even after 50 days, failed to produce atheromata in the aorta, although in some instances deposits of anisotropic fat were found in the liver and adrenals.

In the present experiment no deleterious effects which could be attributed to the feeding of cholesterol were ever displayed by any of the animals. Although the dose of cholesterol administered was a very large one, about 1.6 gm. per kilo of body weight 6 days per week, no evidence of the formation of serious lesions has been obtained. One male which had received this dose of

[8] Anitschkow, N., *Beitr. path. Anat. u. allg. Path.*, 1913, lvi, 379; 1914, lvii, 201.

[9] Chalatow, S. S., *Arch. path. Anat.*, 1912, ccvii, 452; *Beitr. path. Anat. u. allg. Path.*, 1914, lvii, 85.

[10] Bailey, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1914, xii, 68.

[11] Bailey, *Proc. Soc. Exp. Biol. and Med.*, 1915, xiii, 60.

cholesterol for 50 days was examined by Dr. G. Y. Rusk, who was unable to find any lesions in the wall of the aorta, while one female which had received the above dose of cholesterol for 463 days and another which had received it for 511 days were examined by Dr. Bailey who also failed to find any evidence of aortic lesions although deposits of anisotropic fat were found in the liver. That no other acute conditions were caused by the cholesterol feeding may be inferred from the fact that the cholesterol-fed animals displayed normal health throughout the experiment and the percentage of deaths to the date of writing (the animals being about 17 months of age) has not differed appreciably from the percentage of deaths occurring in a like period of time among normal animals.

It is evident, therefore, that the ability of the tissues to dispose adequately of excess of cholesterol varies in different species of animals and is very slight in the rabbit as compared with the guinea-pig, rat, and mouse. That this greater tolerance displayed by mice is not attributable to non-absorption of the cholesterol administered is evident from the very striking effects upon the growth of the animals to which, as we have seen, the administration of cholesterol leads.

Analysis of the Causes of Death.

Between the 10th and 60th weeks two deaths were recorded among the cholesterol-fed males and eight among the cholesterol-fed females. The following is a summary of the causes of death:

Males.

Pneumonia	1
Unidentified cause	1

Females.

Cancer	5
Paratyphoid	1
Pneumonia	1
Unidentified cause	1

SUMMARY.

1. The administration of 40 mg. per day per animal of cholesterol to mice, beginning at 5 weeks after birth (conclusion of the second growth cycle), leads to marked retardation of growth during the earlier portion of the third growth cycle, between the 5th and 10th weeks. From the 10th week onward, however, growth is decidedly accelerated, although the acceleration is insufficient to compensate entirely for the initial retardation.

2. The influence of cholesterol upon the third growth cycle in mice is therefore comparable with that produced by the administration of much smaller doses of tethelin.

3. The influence of cholesterol upon the variability of the animals to which it is fed is, however, directly opposite to the effect exerted by tethelin, for while tethelin reduces the variability of the animals to which it is fed, cholesterol increases their variability. This increase in variability is mainly attributable to the very unequal acceleration, in different individuals, of the latter portion of the third growth cycle.

4. Cholesterol-fed animals do not noticeably differ in build from normal animals. Their coats are smoother and more glossy in appearance than those of normal animals of the same age.

5. Between the 5th and 10th weeks the testicles of cholesterol-fed males becomes very prominent. This disproportion between the size of the testicles and that of the animals bearing them rapidly disappears subsequent to the 10th week. It is probably due to absence of retardation of the growth of the testicles during the first 5 weeks of feeding, so that the retardation of the body growth of the animals leads to disproportionate size of the testicles. The subsequent acceleration of body growth restores the normal proportion.

6. No deleterious effects attributable to the feeding of cholesterol were observed. Three animals which were examined after 50, 463, and 511 days of feeding respectively showed no evidence of lesions in the wall of the aorta such as are observed in rabbits after feeding relatively smaller doses for comparatively brief periods of time.

EXPERIMENTAL STUDIES ON GROWTH.

VI. THE INFLUENCE OF LECITHIN UPON THE GROWTH OF THE WHITE MOUSE.

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(From the Rudolph Spreckels Physiological Laboratory of the University of California, Berkeley.)

(Received for publication, May 25, 1916.)

Previous Knowledge Concerning the Influence of Lecithin upon Growth.

Previous investigations have shown that the effect of lecithin upon growth depends very considerably upon the type and stage of development of the tissue treated. On the one hand King[1] and Johnson[2] found that frog embryos, when fed with egg lecithin grow more rapidly, both in size and in weight, than normal tadpoles, and Bain[3] found that the subcutaneous administration of lecithin leads to a leukocytosis and an increase in the number of red corpuscles, facts which may possibly indicate an increased rate of formation of these tissues. On the other hand Johnson[2] found that lecithin, when administered by mouth, inhibits pigment formation in tadpoles, Browder[4] has shown that the presence of egg lecithin in the culture medium retards the division rate of paramecia, and I have found[5] that egg lecithin, when added to sea water, strongly retards the earlier stages in the development of fertilized sea urchin eggs.

Previous investigations in this laboratory[6] have also shown that lecithin, prepared from egg yolks by extracting them with ether and precipitating with acetone, causes a distinct retarda-

[1] King, H. D., *Biol. Bull.*, 1907, xiii, 40.
[2] Johnson, M., *Univ. California Publications, Zoology*, 1913, xi, 53.
[3] Bain, W., *Lancet*, 1912, i, 918.
[4] Browder, A., *Univ. California Publications, Physiology*, 1915, v, 1.
[5] Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1913, xxxvii, 497.
[6] Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1913, xvii, 344.

tion of the growth of Flexner-Jobling carcinoma in rats, when administered subcutaneously in the neighborhood of the tumor. Lecithin which has been prepared in this manner contains a very considerable proportion of cephalin[7] and possibly a certain admixture of other phospholipins to which the observed effects might conceivably be attributable. We have also found,[8] however, that the lecithin prepared by extracting the anterior lobe of the pituitary body with alcohol, removing the tethelin contained in this extract by precipitation with ether, evaporating the resultant mixture to dryness, taking up the residue in ether, and precipitating with acetone also leads, when administered subcutaneously in localities remote from the tumor, to a decided retardation of the growth of Flexner-Jobling carcinoma. Since cephalin is insoluble in alcohol the lecithin thus prepared must have been free from admixture with cephalin. It is probable, therefore, that the retarding effect of egg lecithin upon the growth of carcinomata is, at least in part, due to lecithin itself.

Description of the Experiments. Methods of Preparing and Administering the Lecithin. Doses Administered.

Mice about 5 weeks of age were taken at random from our stock, transferred to the cages described in the first article of this series,[9] and immediately and thereafter during the entire period of the experiment, fed with lecithin. Thirty-six males and thirty-six females were fed with egg lecithin and twenty-four males with pituitary lecithin. In every respect other than in the administration of the lecithin these animals were handled and fed exactly as were the normal animals.[10] The animals fed with egg lecithin were about 1 month, and those fed with pituitary lecithin about 4 months younger than the normals so that their life and growth were contemporary with the greater part of the life and growth of the normals. The lecithin-fed animals and the normal animals were always weighed in the middle of the afternoon. Unfortunately the average weight of the group of animals taken at random to be fed with egg lecithin was considerably below the

[7] Stern, M., and Thierfelder, H., *Z. physiol. Chem.*, 1907, liii, 370.
[8] Robertson and Burnett, *J. Exp. Med.*, 1916, xxiii, 631.
[9] Robertson, T. B., and Ray, L. A., *J. Biol. Chem.*, 1916, xxiv, 347.
[10] Robertson, *J. Biol. Chem.*, 1916, xxiv, 362.

average weight of normals, the mean defect at 4 and 5 weeks of age being 1.2 gm. in the males and 1 gm. in the females. The reason for this lies in the extremely high variability of normal animals at the age when experimental animals were selected, rendering it not improbable that a limited number of animals chosen at random may deviate somewhat widely from the true average in weight. Thus the variability of the weight of male mice at 4 or 5 weeks of age is over 24 per cent while the mean weight is 12.4 gm.; hence one animal out of every three may be expected to deviate from the average weight by as much as 3 gm. Similarly one female out of every three may be expected to deviate from the average weight by over 2 gm.[11] The abnormality of the animals employed was unfortunately not detected until a late stage in the investigation and it will be necessary, in order to be certain of the validity of the results obtained, to repeat the experiment with a more representative group of animals. However, the results obtained with egg lecithin, in so far as it is possible to interpret them in view of the above facts, are confirmed, as will be seen, by the results obtained with pituitary lecithin and they are therefore tentatively submitted at this time subject to reconsideration in the light of results obtained with a more representative group of animals. Incidentally the difficulty of accurately comparing the growth of these animals with that of normal animals reveals the necessity of ascertaining, before undertaking *comparative* experiments upon growth, that the limited number of animals chosen for the experiment are truly representative of the average.

The animals selected for feeding with pituitary lecithin were slightly, but not to any important extent, supernormal in weight.

The egg lecithin was prepared in the following manner: To the yolk of eggs was added an equal volume of 10 per cent sodium chloride solution and the mixture was well shaken. This mixture was then shaken with an equal volume of ether until it became of a vaseline-like consistency. On standing, in the course of 24 to 48 hours, the ether and water layers separate and the ether may then be pipetted or syphoned off. This ether extract was evaporated, in an open vessel at room temperature, to about one-

[11] Robertson, *J. Biol. Chem.*, 1916, xxiv, 409.

third of its volume, two volumes of acetone were added, and the precipitate was collected, washed once in acetone, drained, heated for a brief period to drive off the major part of the acetone, and then dried at room temperature over sulfuric acid. As stated above, lecithin prepared in this manner contains a considerable proportion of cephalin and possibly other phospholipoids.

6 gm. of this preparation were placed in 60 cc. of the mixed white and yolk of egg and the mixture was allowed to stand on ice over night. At the end of this period the mass of lecithin had attained a soft jelly-like consistency and was easily rubbed up into a uniform emulsion with the egg. 5 cc. of this mixture were supplied daily, except Sundays, to every six mice, so that the daily dose, assuming the material to have been completely consumed, was 83 mg. As a rule the mice of all experimental classes eagerly consumed the egg mixtures supplied to them, but the mice fed with the mixture just described constituted an exception to the rule. They appeared distinctly averse to the mixture and it was only slowly consumed, although in the course of 24 hours, as a rule, but little of it remained uneaten.

The pituitary lecithin was prepared from the supernatant fluid after the precipitation of tethelin from the alcoholic extract of the anterior lobe of the pituitary body.[11] The fluid was evaporated, without exposure to air, until the boiling point reached 78°C. It was then cooled to 0°C. for 24 to 48 hours, which resulted in the deposition of a small amount of material, probably including the greater part of any unprecipitated tethelin, since tethelin is only sparingly soluble in ice-cold alcohol. The fluid decanted from this deposit was then evaporated, without exposure to air, the residue taken up in a small amount of ether, and several volumes of acetone were added to the resultant solution. The precipitate was collected on a hardened filter, washed in acetone, and dried at room temperature over sulfuric acid. The yield was about 20 mg. per anterior lobe or 1.33 per cent of the fresh tissue. 1 gm. of this material was rubbed up in 20 cc. of distilled water, forming a viscous emulsion, and 2 cc. of this were mixed with 20 cc. of egg yolk and white mixture which was divided equally among the twenty-four mice daily, except Sundays. The daily dose was therefore about 4 mg. per animal. This mixture was always quickly and completely consumed.

According to Parke[12] lecithin forms 10.7 per cent of the egg yolk. Assuming the egg yolk to be half the volume of the white, the mixture of white and yolk which was fed to normal animals, and to which the above mentioned quantities of lecithin were added before supplying it to the experimental animals, contained 3.6 per cent of lecithin; that is, 30 mg. per animal per day. The pituitary lecithin added to the egg mixture constituted, therefore, only a trifling addition to the lecithin already present.

Results.—The weights of the animals fed with egg lecithin and those of the normal animals are compared in Tables I and II and graphically illustrated in Figs. 1 and 2, the smooth curves representing the normal growth of white mice during the experimental period and the dotted curves the growth of the animals

TABLE I.

Lecithin-Fed Male Mice.

Age.	Weight.		No. weighed. (Lecithin-fed.)	Age.	Weight.		No. weighed. (Lecithin-fed.)
	Normal.	Lecithin-fed.			Normal.	Lecithin-fed.	
wks.	*gm.*	*gm.*		*wks.*	*gm.*	*gm.*	
4	12.38	10.86	18	25	27.05	24.50	34
5	12.45	11.53	36	26	26.94	25.10	36
6	15.58	13.11	36	27	26.55	24.97	34
7	18.08	15.07	36	28	27.19	25.26	35
8	19.36	16.56	36	29	27.08	25.27	33
9	20.63	17.69	36	30	27.23	25.74	34
10	21.19	19.13	36	32	27.61	26.04	36
11	21.81	20.06	36	34	27.71	26.32	36
12	22.65	20.50	36	36	27.95	26.86	35
13	23.31	21.10	36	38	28.20	26.84	35
14	23.96	21.49	36	40	28.27	27.02	33
15	24.28	22.17	36	42	28.28	27.23	33
16	24.75	22.22	36	44	28.62	27.15	33
17	25.21	22.60	36	46	28.55	27.17	33
18	25.61	23.25	36	48	28.22	27.47	32
19	25.81	23.72	36	50	28.88	27.44	31
20	26.10	24.04	36	52	28.68	27.76	31
21	26.28	24.17	36	54	28.67	28.07	30
22	26.06	24.86	36	56	29.32	27.82	30
23	26.34	24.57	36	58	29.10	27.93	30
24	26.82	24.43	36	60	29.08	27.75	28

[12] Parke, J. L., *Med.-Chem. Untersuch.*, 1866, i, 209.

TABLE II.

Lecithin-Fed Female Mice.

Age.	Weight.		No. weighed. (Lecithin-fed.)	Age.	Weight.		No. weighed. (Lecithin-fed.)
	Normal.	Lecithin-fed.			Normal.	Lecithin-fed.	
wks.	*gm.*	*gm.*		*wks.*	*gm.*	*gm.*	
4	10.39	8.87	15	25	23.79	21.34	35
5	11.81	11.42	31	26	24.04	21.66	35
6	14.12	13.35	36	27	24.00	21.61	35
7	16.77	15.04	36	28	23.58	21.92	33
8	17.99	16.47	36	29	23.84	22.06	35
9	18.78	17.00	36	30	23.92	22.34	29
10	19.38	17.32	36	32	24.18	22.29	34
11	20.04	18.08	36	34	24.18	22.56	33
12	20.31	18.67	36	36	24.65	22.88	33
13	21.04	18.71	35	38	24.80	22.82	33
14	21.21	19.26	35	40	25.03	22.97	33
15	21.78	19.71	35	42	25.07	23.00	33
16	22.14	20.03	35	44	25.52	23.29	33
17	22.29	20.26	35	46	25.68	23.20	32
18	22.22	20.50	35	48	25.45	24.02	32
19	22.60	20.77	35	50	25.50	24.08	31
20	22.60	20.64	35	52	25.76	24.60	31
21	23.06	21.03	35	54	25.78	24.55	29
22	23.32	21.13	35	56	26.00	24.28	29
23	23.51	21.31	35	58	26.26	24.69	29
24	23.68	21.27	35	60	26.12	24.09	28

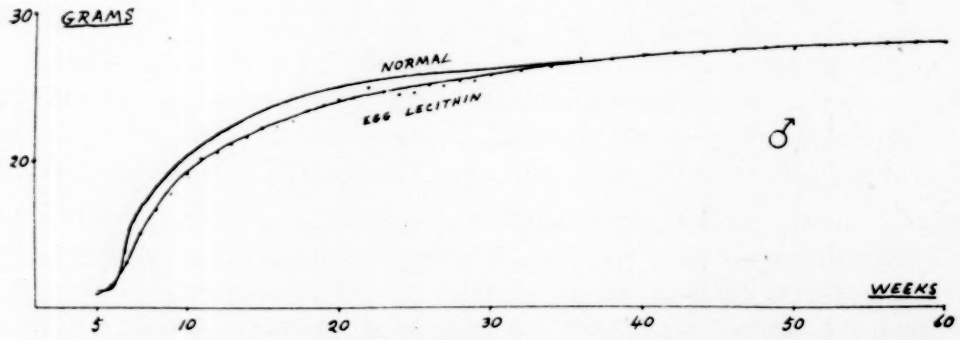

FIG. 1. Comparison of the growth curves of normal males and males fed with lecithin derived from egg yolk. Ordinates of normal curve diminished by 1.2 gm.

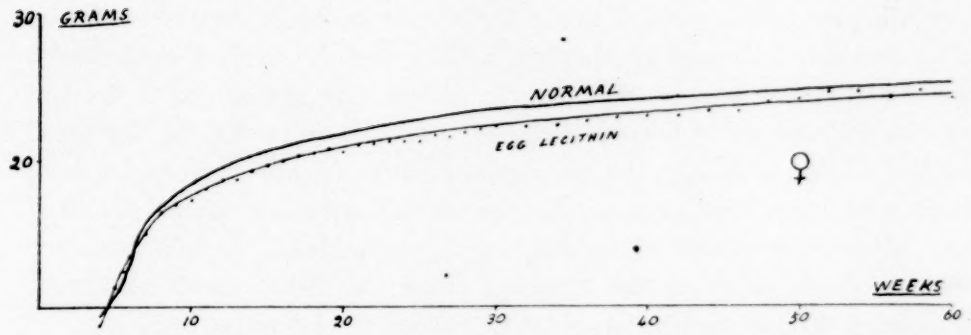

FIG. 2. Comparison of the growth curves of normal females and females fed with lecithin derived from egg yolk. Ordinates of normal curve diminished by 1 gm.

which received lecithin. The dots represent the positions of the actual observations. The ordinates of the normal curves are reduced by 1.2 gm. in the case of the males and 1.0 gm. in the case of the females in order, as far as possible, to compensate for the initial subnormality of the lecithin-fed animals and facilitate a comparison of the two curves.

The effect of the administration of egg lecithin upon the form and proportions of the growth curve is evidently extremely small; a fact which, in view of the very large dosage, is rather surprising. Of course it is uncertain to what extent a uniform reduction of the ordinates of the normal curve is a valid method of representing the normal growth curve of initially subnormal animals; but if it be assumed that this procedure may yield an approximation to the normal curve in such cases, then the only effect of the administration would appear to consist in a very slight—in the case of the males a barely perceptible—retardation of growth without deformation of the curve; *i.e.*, a retardation which is nearly uniform throughout the whole of the third cycle. There appears to be some slight acceleration in the latter part of the cycle in the males, but as this is not evidenced in the females it is probably apparent and not real, resulting from the initial abnormality of the experimental animals.

The variabilities of normal animals and of those fed with egg lecithin are compared in Tables III and IV and Figs. 3 and 4. Here again we see very little effect of the administration when the necessary allowance is made for initial differences in the

variabilities of the normal and the experimental groups of animals. The lecithin-fed animals, being initially lighter than the normals, were also initially less variable.[13] When the initial point on the variability curve of the lecithin-fed animals is raised to the level of the initial point on the variability curve of the normal animals it is seen that the administration of egg lecithin caused little if any alteration in the variability of the males and a slight increase in the variability of the females from the 30th week onwards, an effect which, again, may be apparent and not real.

TABLE III.

Lecithin-Fed Male Mice.

Age.	Variability.		Age.	Variability.	
	Normal.	Lecithin-fed.		Normal.	Lecithin-fed.
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cent*
4	24.4	23.1	25	11.0	8.6
5	24.6	23.2	26	10.8	8.4
6	22.0	21.7	27	11.3	7.4
7	16.9	19.4	28	10.9	8.8
8	15.9	18.5	29	10.9	8.8
9	16.5	15.8	30	9.5	8.8
10	16.7	13.7	32	9.6	8.8
11	13.3	11.4	34	10.5	8.5
12	13.7	10.2	36	10.1	8.8
13	14.2	9.8	38	10.5	8.8
14	14.1	9.4	40	10.4	8.3
15	11.9	9.9	42	11.2	9.5
16	12.7	8.9	44	12.0	10.0
17	12.4	8.5	46	11.7	10.1
18	12.1	8.7	48	10.8	9.1
19	12.1	7.5	50	11.1	9.7
20	10.8	8.0	52	10.7	9.3
21	11.2	8.2	54	12.0	8.7
22	9.0	7.6	56	12.0	9.0
23	9.8	7.9	58	10.9	7.8
24	10.1	8.7	60	11.8	8.1

[13] Robertson, *J. Biol. Chem.*, 1916, xxiv, 372, 392, 405.

TABLE IV.

Lecithin-Fed Female Mice.

Age.	Variability.		Age.	Variability.	
	Normal.	Lecithin-fed.		Normal.	Lecithin-fed.
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cent*
4	23.4	16.3	25	11.4	9.4
5	19.4	15.8	26	11.6	8.9
6	18.9	12.7	27	11.8	9.8
7	15.0	9.7	28	10.3	9.8
8	13.9	8.8	29	12.2	10.2
9	13.4	8.6	30	12.4	10.6
10	14.3	9.6	32	11.5	11.2
11	12.6	8.2	34	12.2	10.9
12	12.6	8.6	36	11.2	10.8
13	13.0	8.2	38	12.3	11.4
14	13.2	7.6	40	11.9	11.2
15	13.7	8.9	42	13.1	12.1
16	12.6	8.4	44	15.3	12.9
17	11.9	8.5	46	14.6	13.6
18	11.9	9.0	48	14.6	14.2
19	12.0	8.6	50	14.0	14.4
20	11.2	8.5	52	15.3	14.0
21	12.3	8.8	54	15.8	14.7
22	12.5	9.1	56	16.5	14.1
23	12.0	9.0	58	16.7	13.5
24	11.3	9.4	60	17.8	13.1

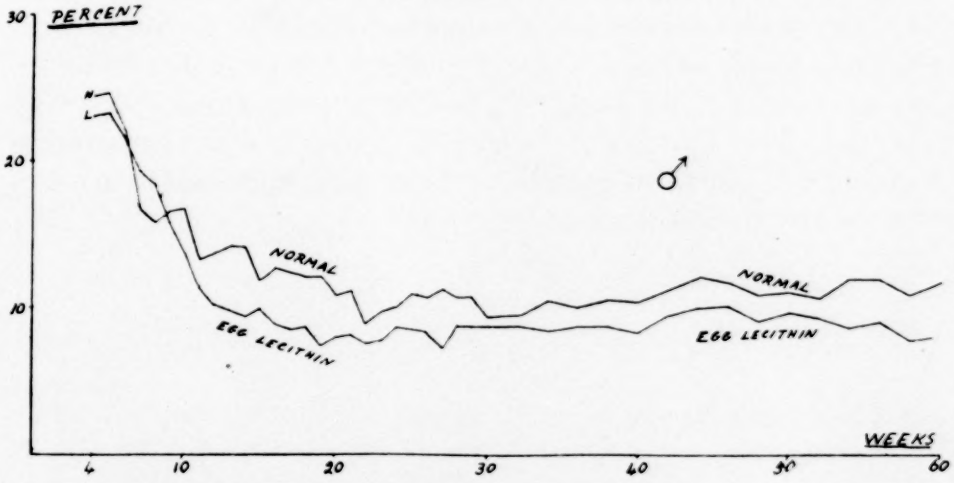

FIG. 3. Comparison of the variability curves of normal males and males fed with lecithin derived from egg yolk.

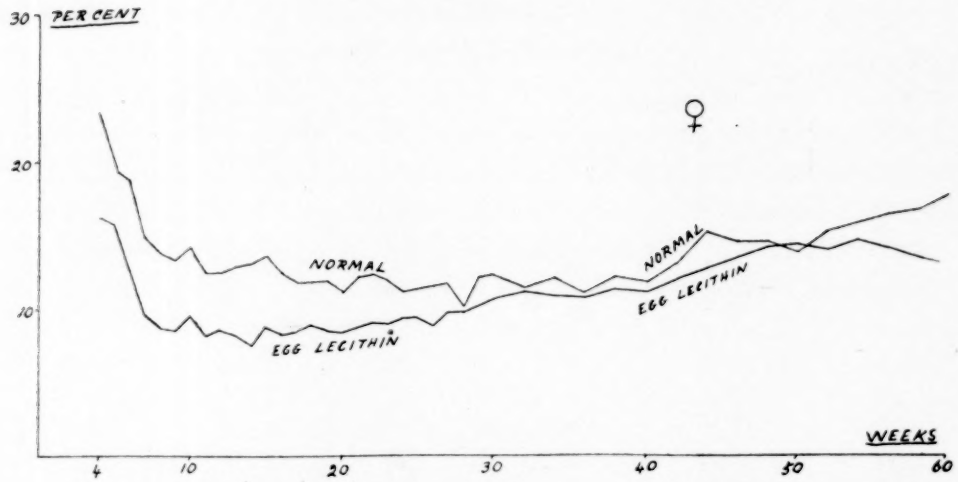

FIG. 4. Comparison of the variability curves of normal females and females fed with lecithin derived from egg yolk.

The weights of the animals fed with pituitary lecithin and those of normal animals are compared in Table V and graphically illustrated in Fig. 5. It will be seen that the lecithin-fed animals were initially slightly supernormal in weight but that within 2 weeks of commencing the feeding of lecithin they dropped slightly behind the normals in weight, remaining slightly and nearly uniformly subnormal in weight throughout the experiment. Apparently the administration of pituitary lecithin caused a slight and nearly uniform retardation of weight and no acceleration, unless the slight deviations from the smoothed curve at 10 weeks indicate some acceleration. These deviations are so slight, however, that no certain conclusions can be drawn from them. We may infer, therefore, that pituitary lecithin causes a slight retardation of growth in the third growth cycle and no appreciable acceleration at any period during the cycle.

TABLE V.

Male Mice Fed with Pituitary Lecithin.

Age.	Weight.		No. weighed. (Lecithin-fed.)	Age.	Weight.		No. weighed. (Lecithin-fed.)
	Normal.	Lecithin-fed.			Normal.	Lecithin-fed.	
wks.	*gm.*	*gm.*		*wks.*	*gm.*	*gm.*	
5	12.45	14.63	15	26	26.94	25.89	22
6	15.58	15.54	24	27	26.55	25.70	23
7	18.08	16.77	24	28	27.19	26.14	22
8	19.36	17.92	24	29	27.08	26.09	23
9	20.63	17.92	24	30	27.23	26.06	18
10	21.19	19.10	24	32	27.61	26.72	23
11	21.81	19.06	24	34	27.71	26.48	23
12	22.65	20.46	24	36	27.95	26.54	23
13	23.31	21.38	24	38	28.20	26.91	23
14	23.96	22.58	24	40	28.27	27.17	23
15	24.28	23.23	24	42	28.28	27.76	23
16	24.75	23.73	24	44	28.62	27.85	23
17	25.21	24.23	24	46	28.55	27.85	23
18	25.61	24.48	23	48	28.22	28.11	22
19	25.81	24.71	24	50	28.88	28.00	22
20	26.10	25.15	23	52	28.68	28.02	22
21	26.28	25.04	23	54	28.67	27.84	22
22	26.06	25.61	23	56	29.32	27.85	20
23	26.34	25.35	23	58	29.10	28.00	16
24	26.82	25.65	23	60	29.08	28.19	16
25	27.05	26.02	23				

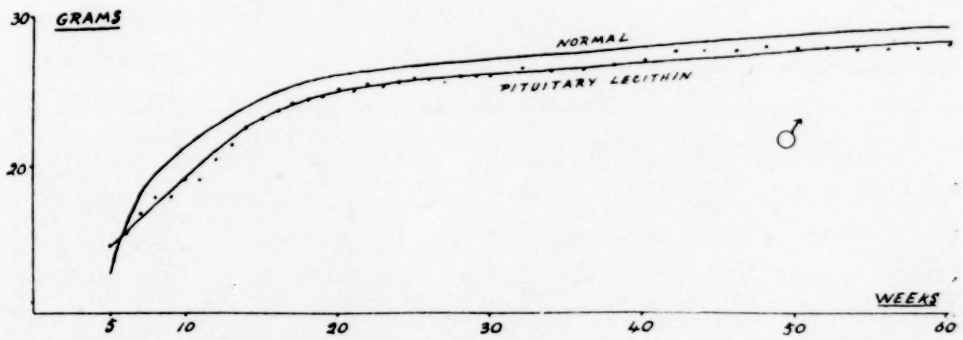

FIG. 5. Comparison of the growth curves of normal males and males fed with lecithin derived from the anterior lobe of the pituitary body.

The variabilities of the animals fed with pituitary lecithin and those of normals are compared in Table VI and Fig. 6. It will be seen that the administration of pituitary lecithin caused an evident diminution of variability.

TABLE VI.

Male Mice Fed with Pituitary Lecithin.

Age.	Variability.		Age.	Variability.	
	Normal.	Lecithin-fed.		Normal.	Lecithin-fed.
wks.	*per cent*	*per cent*	*wks.*	*per cent*	*per cent*
5	24.6	24.6	26	10.8	7.2
6	22.0	19.7	27	11.3	7.4
7	16.9	17.1	28	10.9	7.1
8	15.9	13.9	29	10.9	7.4
9	16.5	15.1	30	9.5	7.3
10	16.7	12.6	32	9.6	7.4
11	13.3	13.4	34	10.5	7.6
12	13.7	13.7	36	10.1	7.5
13	14.2	12.3	38	10.5	7.5
14	14.1	10.8	40	10.4	7.8
15	11.9	9.2	42	11.2	7.5
16	12.7	8.8	44	12.0	7.6
17	12.4	7.8	46	11.7	7.5
18	12.1	7.3	48	10.8	7.3
19	12.1	8.3	50	11.1	7.9
20	10.8	6.9	52	10.7	7.9
21	11.2	7.2	54	12.0	7.6
22	9.0	6.8	56	12.0	8.6
23	9.8	6.8	58	10.9	7.6
24	10.1	7.0	60	11.8	7.7
25	11.0	6.4			

Considering the much smaller dose administered, pituitary lecithin would appear to exert a considerably greater effect upon the growth of mice than egg lecithin. Whether this disparity of effect is attributable to the initial subnormality of the animals fed with the egg lecithin, or to an admixture of other and more potent phospholipoids with the pituitary lecithin it is impossible at present to assert. Having regard, however, to the fact that the pituitary lecithin comprised only a small proportion of the lecithin present in the egg mixture which was administered to the animals, even the slight effects observed are probably to be

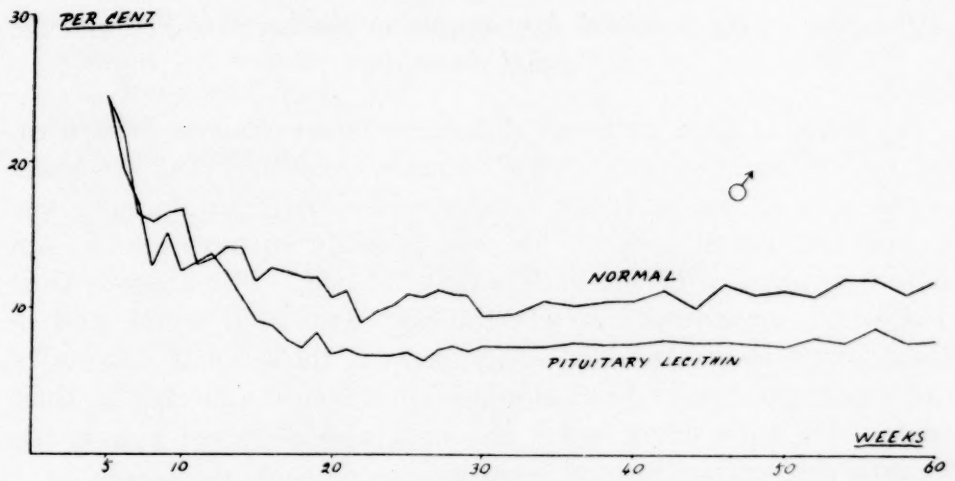

FIG. 6. Comparison of the variability curves of normal males and males fed with lecithin derived from the anterior lobe of the pituitary body.

attributed to admixture of some other substance, possibly a trace of tethelin, or at all events to some peculiarity of lecithin prepared from the anterior lobe of the pituitary body.

Summarising the results, it is evident that the effect of lecithin upon the growth of white mice is much less than might be expected from the very decided action exerted by lecithin upon the growth of carcinomata, the divisions of paramecia, and the growth of tadpoles. The reason for the lack of action when administered by mouth to mice probably lies in the fact that lecithin is very readily split by lipase and is therefore, in all probability, not absorbed as such to any appreciable extent. In the experiments on carcinoma which I have cited the lecithin was administered subcutaneously and was therefore for some time present in the tissues as such, and the absorption by paramecia or tadpoles may not improbably differ from absorption in the alimentary canal of a mammal. The observed retarding action may possibly be attributable to the injurious effects of the absorption of excess of choline.

Differences in the External Appearance of the Lecithin-Fed and the Normal Animals.

No very evident external differences were observed between the normal and the lecithin-fed animals, excepting that the coats of the animals fed with egg lecithin presented from an early age a wretched appearance. This was possibly in part due to the slow consumption of the daily supply of egg. The animals thus had ample opportunity to get the egg upon their coats, and as the mixture was extremely sticky many of the animals constantly had small patches of dried egg-lecithin mixture adhering to their coats. No such effect upon the coat was observed among the animals fed with pituitary lecithin.

All of the animals fed with lecithin displayed an unusually high degree of susceptibility to infection. Among the males fed with egg lecithin seriously infected wounds were common, resulting from injuries received in fighting, while among the animals fed with pituitary lecithin a form of infection of the respiratory tract was met with, accompanied by sneezing, gurgling noises in the throat, and progressive slow loss of weight. No similar cases were encountered in any of the other experimental groups, but it repeatedly appeared in this group subsequent to the 50th week, despite the fact that animals suspected of infection were isolated until their condition became evident and were then killed, and the food vessels, nests, and cage were repeatedly washed with alcohol.

Analysis of the Causes of Death.

Between the 10th and the 60th weeks seven deaths were recorded among the males and seven among the females which were fed with egg lecithin. During the same period eight deaths were recorded among the animals which were fed with pituitary lecithin. The following is a summary of the causes of death:

Males fed with egg lecithin.

Cancer	1
Gastric ulcer	1
Infected wounds received while fighting	4
Unidentified cause	1

Females fed with egg lecithin.

Cancer	1
Duodenal ulcer	1
Abscess	1
Infected injury	1
Acute nephritis	1
Unidentified causes	2

Males fed with pituitary lecithin.

Unidentified infection of respiratory tract[14]	7
Unidentified cause	1

SUMMARY.

1. The administration by mouth of 80 mg. per day per animal of egg lecithin, beginning at 4 weeks after birth (conclusion of the second growth cycle), leads to no deformation of the curve of growth, the only demonstrable effects of the administration consisting in a very slight uniform retardation of growth and a low degree of resistance to infection, both effects being not improbably attributable to the injurious action of excess of choline absorbed from the alimentary tract.

2. The administration by mouth of 4 mg. per day of lecithin derived from the anterior lobe of the pituitary body produces similar effects. Having regard to the comparatively small dose administered it is possible that these effects may in part have been due to admixture of other and more potent substances with lecithin derived from this source or at all events to a peculiarity of lecithin derived from the anterior lobe of the pituitary body.

3. The lack of effect of lecithin administered by mouth in comparison with its effects when administered subcutaneously or to lower organisms is probably attributable to the fact that lecithin is completely split during digestion and is not absorbed to any appreciable extent as such.

[14] One of these animals, in postmortem examination, was found to be suffering from cancer.

EXPERIMENTAL STUDIES ON GROWTH.

VII. THE INFLUENCE OF THE ADMINISTRATION OF EGG LECITHIN AND OF CHOLESTEROL TO THE MOTHER, UPON THE GROWTH OF SUCKLING MICE.

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Object of the Experiments.

While lecithin has been shown to exert a retarding action upon certain types of growth, such as the early stages of the development of sea urchin embryos,[1] the growth of carcinoma,[2] and the division rate of paramecia,[3] it has also been found to accelerate certain other types of growth, such as that leading to the production of leukocytes and erythrocytes[4] and the development of tadpoles.[5] Similarly it has been found that cholesterol, while very markedly retarding the initial portions of the third growth cycle in mice, nevertheless accelerates the later growth of mice;[6] also the growth of carcinomata[7] and the division rate of paramecia.[3] It is evident therefore that these substances may exert an effect upon one type of growth which is exactly the reverse of their effect upon another type of growth, and, furthermore, that as a general rule the effect of cholesterol upon a given type of growth is opposite to that exerted by lecithin upon the same type of growth.

[1] Robertson, T. B., *Arch. Entwcklngsmechn. Organ.*, 1913, xxxvii, 497.

[2] Robertson, T. B., and Burnett, T. C., *J. Exp. Med.*, 1913, xvii, 344.

[3] Browder, A., *Univ. California Publications, Physiology*, 1915, v, 1.

[4] Bain, W., *Lancet*, 1912, i, 918.

[5] King, H. D., *Biol. Bull.*, 1907, xiii, 40. Johnson, M., *Univ. California Publications, Zoology*, 1913, xi, 53.

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The effects of these substances when administered by mouth upon the third growth cycle in mice (the growth subsequent to 4 or 5 weeks of age) have been discussed in the two preceding articles of this series. We have sought to extend these results by investigating the effects of lecithin and cholesterol upon the growth during the latter 2 weeks of the first growth cycle and the 1st week of the second growth cycle; that is, upon the growth of mice during the first 3 weeks succeeding birth. The young during this period, or at all events during the first 2 weeks of this period, are dependent upon the mother for their nutrition.[8] As the hypodermic administration of these substances to such young animals presents very great technical difficulties and the necessary manipulations might exert an uncertain effect upon the welfare of the animals, we have sought to influence the growth of the young through administration of lecithin or cholesterol by mouth to the mothers, fully recognising, however, that the interpretation of the results obtained is complicated by the possibility that the quantity and quality of the mother's milk may be directly affected by these substances, and that the growth of the young may in this way be indirectly affected, independently of direct transmission of the substances to the young through the medium of the mother's milk.

Methods.

Twenty-five litters of mice were divided into three groups, A, B, and C. The division was made at the birth of the litters, the litters being alternated so that the first litter came in group A, the second in group B, the third in group C, and so on. In this way the groups obtained consisted of nearly equal numbers of initially similar animals. The litters were kept in separate cages during the period of the experiment and the mother was supplied with an abundance of rolled barley and water, and occasionally with fresh lettuce leaves. In addition to this the control animals (group A) each received daily 1 cc. of mixed yolk and white of egg, another group of animals (B) received the same amount of egg mixture to which, however, were added 100 mg. of egg lecithin, prepared in the manner described in the preceding article,

[8] Robertson, *J. Biol. Chem.*, 1916, xxiv, 373.

while the third group received the same amount of egg mixture to which were added 100 mg. of Merck's cholesterol. The animals were weighed daily or every 2nd day to the nearest cg., each mouse in the litters being weighed separately. The litters were all kept in the same room and under identical conditions.

RESULTS.

The results obtained are shown in Table I and depicted graphically in Figs. 1 and 2. It will be seen that the administration of egg lecithin to the mother produced a slight and nearly uniform retardation in the growth of the young, in keeping with its effect upon the later growth of mice when administered by mouth.[9] The administration of cholesterol to the mother, however, resulted in a much more profound retardation of the growth of the young,

TABLE I.

Age.	Normal.		Lecithin-fed.		Cholesterol-fed.	
	No. weighed.	Average weight.	No. weighed.	Average weight.	No. weighed.	Average weight.
days		*gm.*		*gm.*		*gm.*
Birth.	48	1.47	45	1.27	47	1.30
1	40	1.40	21	1.70	30	1.61
2	26	1.80	41	1.85	39	1.75
3	24	2.27	19	1.96	32	1.98
4	19	2.70	29	2.49	19	2.37
5	19	2.86	27	2.83	30	2.59
6	29	2.60	16	2.85	25	2.83
7	18	2.99	34	3.24	26	3.19
8	30	3.80	27	3.33	25	3.58
9	25	3.84	21	3.71	24	3.60
10	29	4.43	19	4.04	21	3.84
11	26	4.67	21	4.13	18	4.00
12	37	4.50	23	4.30	26	4.14
13	23	4.77	11	4.80	18	4.21
14	26	5.09	27	4.81	26	4.36
15	32	5.51	16	4.62	21	4.25
16	25	5.50	19	5.10	23	4.38
17	26	5.67	22	5.51	14	4.54
18	28	6.51	12	6.17	15	4.64
19	18	7.19	20	6.28	25	5.36
20	25	7.59	20	6.62	25	6.02

[9] Robertson, *J. Biol. Chem.*, 1916, xxv, 647.

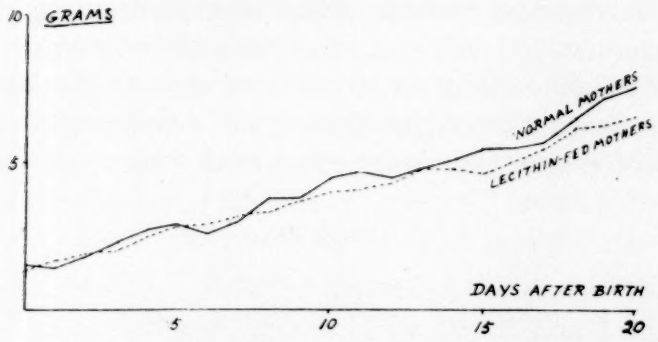

FIG. 1. Comparison of the growth of mice suckled by normal mothers what that of mice suckled by mothers fed with lecithin derived from egg yolk.

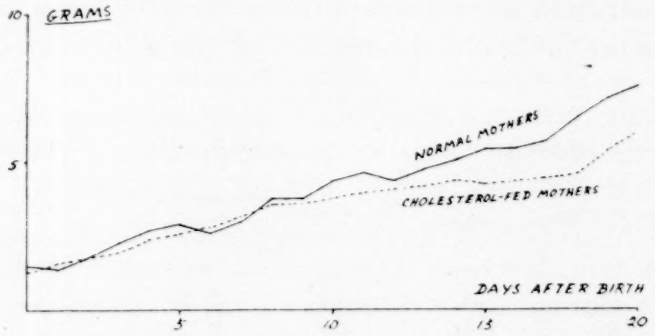

FIG. 2. Comparison of the growth of mice suckled by normal mothers with that of mice suckled by mothers fed with cholesterol.

an effect which was manifestly determined by the stage of development of the animals, for prior to the 9th day very little if any retardation of growth occurred, but thereafter, until the termination of the period of lactation on the 20th day after birth, accumulative and very decided retardation of the growth of the young was observed, with the result that by the 20th day after birth the sucklings of the cholesterol-fed mothers were 1.57 gm. or 21 per cent lighter than the young of the normal group. It would appear that cholesterol, when administered in this way, brings about retardation of the latter portion of the first and the initial portion of the second growth cycle,[8] but whether this effect is attributable to transmission of excess of cholesterol to the young through the medium of the mother's milk or to interference with the quantity or quality of milk supplied, our results do not as yet permit us to decide.

SUMMARY.

1. The administration of 100 mg. of egg lecithin per day by mouth to the mother slightly retards the growth of suckling mice.

2. The administration of 100 mg. of cholesterol per day by mouth to the mother causes a very marked retardation of the growth of suckling mice between the 9th and 21st days after birth.

3. Our experiments do not enable us to decide whether these actions represent the direct effect of lecithin and cholesterol upon the growth of sucklings or only an indirect effect due to interference with the supply of milk.

AN IMPROVED HASSELBALCH HYDROGEN ELECTRODE AND A COMBINED TONOMETER AND HYDROGEN ELECTRODE, TOGETHER WITH RAPID METHODS OF DETERMINING THE BUFFER VALUE OF BLOOD.*

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CONTENTS.

1. The two compartment hydrogen electrode..... 669
2. The hydrogen ion concentration of blood and serum..... 672
3. The combined tonometer and hydrogen electrode..... 675
4. Charts for finding the buffer value of blood and serum..... 677
5. The indicator method for determining the buffer value..... 679
6. The indicator method for P_H of stomach contents..... 680

1. The Two Compartment Hydrogen Electrode.

After experimenting for a year with hydrogen electrodes based on the form designed by Michaelis, the senior author attempted to modify Hasselbalch's electrode so as to eliminate the danger of loss of CO_2 during transfer of solution. The electrode vessel (Fig. 1) has two compartments, the smaller of which contains the electrode proper. Hydrogen is shaken with a portion of the solution in the large compartment and then passed through the large middle stop-cock into the smaller compartment containing another portion of the solution, where the reading is made. Stop-cock *a* is not greased and conducts electrolytically while closed. Since the hydrogen is brought to an equilibrium with the first portion of the solution, it does not appreciably remove CO_2 from the second portion. The lower the buffer value of the

* The Apparatus was bought out of the Research Fund of the Graduate School.

solution, the larger the large compartment should be. If the two compartments are the same size, fairly accurate readings may be made on blood, but if the samples are not exceedingly small it is better to have the ratio of the compartments as in Fig. 1. A demonstration of the reliability of this electrode was made by charging it with blood serum and making a succession of readings from the end of 5 minutes until the end of 48 hours; they were the same within 1 millivolt. The potentiometer and Weston cell were compared with ones recently calibrated by the Bureau of Standards.

A description of the technique of determinations on blood will suggest the precautions necessary for any solution. The blood

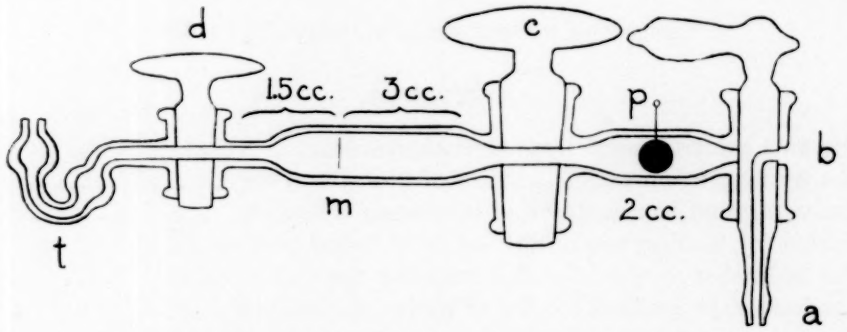

FIG. 1. Two compartment hydrogen electrode. Hydrogen is shaken with one portion of the blood in the large compartment and passed into another portion of the blood in the small compartment for the measurement of the electrode potential.

is collected as described by McClendon (1916 a), and the defibrinated blood or serum used, or it is passed directly into the electrode by means of a rubber tube connected to the needle or cannula in the blood vessel. In the latter case, the electrode must contain some hirudin or isotonic, neutralized sodium oxalate. The dilution of blood with an equal volume of a neutral solution of low buffer value has been shown by Michaelis, and more recently by Corral, to have no perceptible effect on the reaction.

In order to obtain absolute values, the electrode must be cleaned with potassium bichromate in H_2SO_4 and replatinized a few seconds by removing the stop-cock *a* and inserting a platinum wire anode through the opening *b* (Fig. 1). It is then thor-

oughly rinsed with distilled water (and the stop-cock *a* lubricated with serum or KCl and glycerol) and filled with pure hydrogen; a drop of water in the trap *t* serving to prevent the backward diffusion of air until all stop-cocks are closed. The rubber tube admitting the blood is connected to *a*, and the air in it allowed to pass out at *b*, after which this stop-cock is turned so as to admit the blood into the electrode, when the other stop-cocks are opened. The blood is allowed to enter until it reaches the mark *m*, the apparatus then containing 5 cc. of blood and 1.5 cc. of hydrogen.

All the stop-cocks are closed and the apparatus is shaken or inverted 200 times. The middle stop-cock is opened and the apparatus tapped or swung so as to cause the hydrogen to pass into the smaller compartment by displacing part of the blood. This backflowing blood helps to complete the equilibration of the hydrogen in regard to CO_2. The smaller compartment now contains 1.5 cc. of hydrogen and 0.5 cc. of blood, and is shaken or inverted 200 times; and the stop-cock at *a* is immersed in the KCl solution connected with the calomel electrode. The connection is completed by hooking a wire in the platinum loop *p*, the wire being bright and weighted sufficiently to maintain a good contact.

If there is but little oxygen in the blood, the definitive reading should be obtained immediately (provided it is the same temperature as the calomel electrode), but if it contains much oxyhemoglobin the reading will be too low, and will slowly rise as the oxygen combines with the hydrogen in the platinum black. If more than 15 minutes are allowed to elapse, the electrode should be shaken in order to bring a fresh layer of blood into contact with the platinum before another reading. It is not necessary to shake continuously, as recommended by Hasselbalch, but continuous shaking hastens the disappearance of the oxygen. If much hydrogen disappears by combination with the oxygen, it is necessary to restore atmospheric pressure by momentarily opening the stop-cocks *c* and *d*, but this cannot be done more than once without danger of admitting oxygen from the air.

Since convenience and accuracy depend on the details of the construction of the electrode, a few words as to its manufacture may be permitted. If platinum is used for the electrode proper,

it must be exceedingly thin so as not to absorb an appreciable amount of hydrogen. Gold is much better, but must be attached to a platinum wire fused through the glass. Platinum may be welded to platinum at a white heat, but gold melts at this temperature. It is better to melt a drop of gold on the end of the platinum wire and hammer this drop (after cooling) to the gold disc. In doing this, a gold wire or scrap of foil is wrapped around the end of the platinum wire and held in the flame until it is just melted. If held in the flame longer, the gold will amalgamate with the platinum, forming a brittle alloy.

The bore of stop-cock *c* should be at least 3 mm., as the larger it is the more easily the hydrogen is caused to pass into the small compartment.[1]

2. The Hydrogen Ion Concentration of Blood and Serum.

It is stated by Hasselbalch and others that serum is more alkaline than blood. Hasselbalch found that the less oxygen blood contained, the more alkaline it appeared to be when measured by the hydrogen electrode. Since hemoglobin may carry a large store of oxygen, the discrepancy between the reaction of blood and serum is apparently due to faulty technique. Although it is theoretically impossible for the mere proximity of the corpuscles to change the reaction of the plasma, and Michaelis claims that coagulation does not affect the reaction, it seemed advisable to test this question experimentally. The result was that the reaction of serum and defibrinated blood from the same source and without loss of CO_2 is the same. This tells nothing concerning the reaction of the interior of the corpuscles or the effect of laking on the reaction of the blood, which will be considered in a later paper.

Hasselbalch states that the P_H ($= -\log H^{\cdot}$) of blood is 0.17 lower at 37° than at 19°, and Michaelis states that P_H determinations at 37° are 0.21 lower than the average of determinations on different persons at room temperature. Since Corral and others have not observed high temperature coefficients for the hydrogen

[1] The best electrodes were blown by A. S. Jones, 62 Alexander St., Princeton, N. J., who also made the stop-cocks, and the tonometers to be described.

ion concentration of various buffer mixtures, it seemed strange that the blood should be an exception. In order to test this, two saturated KCl calomel electrodes were compared at the same temperature and found to be alike. One of them was placed in a thermostat at 37° and allowed to remain 3 days, since Wolff found it took a long time to come to equilibrium. Blood and serum were tested at 23° and at 37° against these calomel electrodes. The difference in the P_H at the two temperatures was not greater than 0.02 and was therefore within the limit of experimental error. The same was found true of $NaHCO_3$ solutions. This experiment was repeated with 0.1 N KCl calomel electrodes. The P_H was lower at the higher temperature, but the difference did not exceed 0.01 to 0.07 when corrected for the increased vapor tension of water.

The reason for using two types of calomel electrodes is that the result depends on the value of the E. M. F. of the calomel electrode against the normal hydrogen electrode, as calculated by different investigators. In such calculations the hydrogen ion concentration in the hydrogen electrode is determined by electric conductivity or other indirect means. Ellis observed that the H ion concentration of HCl, even at 0.005 N, is different, when calculated from conductivity data, from the value obtained by electrode potential. For this reason the results obtained on blood at different temperatures are not to be considered absolute, but merely reproducible by using the ordinarily accepted data, as given by McClendon (1916 a, Fig. 5) and Michaelis.

Since the viscosity of water decreases with rise of temperature the dissociation of acids, calculated from conductivity data, is erroneous unless these data are corrected for viscosity. Owing to the increased dissociation at higher temperature, the P_H of pure water is about 0.3 less at 37° than at 18°, but the P_H of an alkaline solution should be greater at the higher temperature. We found the P_H of N $NaHCO_3$ to be 7.9, the P_H of 0.1 N solution, which is dissociated (hydrolytically) to a greater extent, to be 8.3. The latter result confirms that of Auerbach and Pick. Apparently the effect of rise in temperature increasing the dissociation of water and thus decreasing the P_H is partly counterbalanced by the greater dissociation of alkaline salts, increasing the P_H, so that the reaction of the blood remains nearly the same when

measured by means of the hydrogen electrode at different temperatures. The dissociation of water and hence the hydroxyl ion concentration in the blood increase with rise in temperature; hence the blood becomes more alkaline with rise in temperature, as pointed out by Höber.

Hasselbalch has shown that the P_H of arterial blood (*i.e.*, at alveolar CO_2 tension) is remarkably constant. On the contrary, Menten and Crile claim that the blood returning from different organs shows great differences in P_H. The unpublished determinations which the senior author made for Dr. Uhlrich, using electrodes previously described, showed variations in the P_H of venous blood. The average P_H of venous blood is less than 0.05 lower than that of arterial blood, and smaller fluctuations in the CO_2 content must necessarily have less effect on the reaction. The records in the literature of the CO_2 content of blood returning from various organs are very discordant. According to Hill and Nabarro, activity of an organ sometimes reduces the CO_2 content of its venous blood because the effect of increased metabolism is overcompensated by vascular dilatation. Perhaps the chief cause in the variations in the P_H of venous blood is the variation in the blood flow, and hence stasis attending the collection, which is more or less unavoidable in the human subject, is a real source of error.

It has been thoroughly established that changes in the H ion concentration of the blood affect the respiratory center, but for those who hope to detect a change in the reaction of the blood during dyspnea, a little calculation may be of interest. Haldane and Priestley observed that a rise of 0.2 per cent in the CO_2 of the alveolar air doubles lung ventilation. The change in the H ion concentration of the blood caused by a rise in the CO_2 tension of 0.2 per cent of an atmosphere is far within the limits of error of the gas chain method as applied to blood. Hence measurable changes in the P_H of arterial blood mean changes in the threshold for stimulation of the respiratory center as in shock or after drugs.

The respiratory center is apparently not so sensitive to the infusion of HCl into the blood, but this may be due to a difference in permeability and concentration gradient. Since CO_2 is produced by the respiratory center, an increase in this gas in the blood

would decrease the outward diffusion from the center, which would become less alkaline. A mere increase in the H ion concentration of the blood would have much less effect on the diffusion of CO_2, since only a minute fraction of the latter is dissociated. If acid were injected until free acid appeared in the blood, some would diffuse into the respiratory center. Judging by experiments on other cells, fatty acids should diffuse in faster than mineral acids, and hence be more effective in increasing respiration, as is actually found to be the case.

When HCl is added to blood *in vitro*, its content of free CO_2 is increased, but it is incorrect to suppose that the same final result is obtained by infusion of HCl. In the latter case the respiratory center is stimulated and the CO_2 eliminated, so that in a short time the CO_2 tension of the blood is below normal. Since the content of the blood in free CO_2 varies directly with the CO_2 tension, the infusion of HCl reduces the free CO_2 in the blood. The reduction is not sufficient, however, to maintain the normal reaction of the blood, as first shown by Szili.

Owing to the impossibility of measuring the H ion concentration of the respiratory center itself, all factors with a possible influence on it should be studied. For this reason attention is called, in the next section, to the buffer value.

The normal P_H of venous blood seems to fluctuate about 7.5, and that of arterial blood to be a little higher, but the difference is not usually measurable. The number of determinations have been too few, however, to separate individual variations entirely from errors, and no absolute values can be given.

3. The Combined Tonometer and Hydrogen Electrode.

A combined tonometer and hydrogen electrode was described by Peters, but the large rubber stopper rendered it objectionable. The form shown in Fig. 2 has been found convenient and reliable. The gold disc with platinum wire *p* is sealed in the 2 cc. compartment, and the stop-cock *c* is lubricated with a conducting solution and immersed in the KCl trough so that this part of the apparatus can be used as a hydrogen electrode in the same manner as the form shown in Fig 1. The description of the manipulation of the apparatus for blood is the same as for any other fluid. The blood is admitted at *a* until it completely fills the

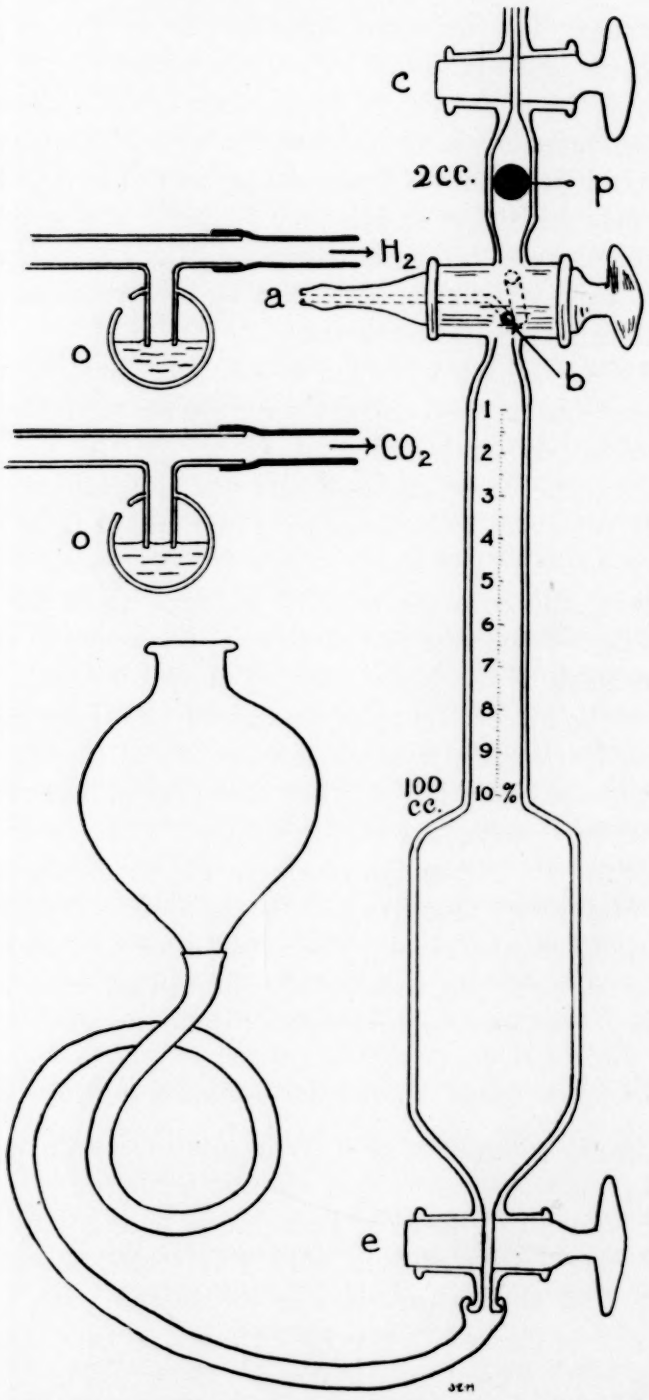

FIG. 2. Combined tonometer and hydrogen electrode. Blood is passed from the 2 cc. compartment into the 100 cc. tonometer and back into the 2 cc. compartment, where the electrode potential is determined.

2 cc. compartment, and the stop-cocks are closed. The apparatus is set up vertically and a rubber tube from a mercury funnel attached to the lower end *e*. By raising the funnel, the 100 cc. tonometer is filled with mercury which forces the air out at *a*. The tubes from the hydrogen and CO_2 generators are provided with overflows *o* to keep these gases at constant and approximately atmospheric pressure. The CO_2 is admitted at *a*, and the stop-cock *a* turned so as to force out the contained air at the side opening *b*, and then turned back so as to allow the CO_2 to enter the tonometer when the funnel is lowered. When the required per cent of CO_2 is read off on the graduations of the tonometer, the stop-cocks *e* and *a* are closed, the hydrogen tube is connected to *a*, the air washed out at *b*, the tonometer filled with this gas, and *e* closed. The stop-cock *a* is so turned as to connect the 2 cc. chamber with the tonometer by means of a hole that is at least of 3 mm. bore. By tapping or swinging the apparatus, the blood is shaken down into the tonometer, which is rotated on its long axis (placed horizontally) by means of a rubber band passed around a revolving axle above it. About 0.5 cc. of the blood is shaken back into the 2 cc. chamber, *c* immersed in the KCl solution, *p* connected to a wire, and the reading taken in the usual manner.

The remainder of the blood in the tonometer is forced into the 2 cc. chamber by means of mercury, and the second CO_2 mixture made in the tonometer in the same manner as the first. The same sample of blood will do for a series of determinations provided the mercury is pure. Before using the apparatus, it should be prepared in the same manner as the electrode shown in Fig. 1.

4. Charts for Finding the Buffer Value of Blood and Serum.

In a previous paper (McClendon, 1916 a) the buffer value was indicated by the difference between the P_H at 3 per cent and at 10 per cent CO_2 tension. The difference in this index of the buffer value for different bloods is so small as to make the charts shown in Fig. 3 desirable as aids in its determination.[2] They will at

[2] The quantity of data used in the making of these charts is not as large as might be desired, but the fact that the junior author cannot continue the work makes it necessary to publish them in their present form.

least serve as guides in the construction of similar charts by investigators engaged in similar work. The data on blood are not in disagreement with the limited observations of Hasselbalch, but no comparative data on serum were found in the literature. The P_H values found by Höber are too low.

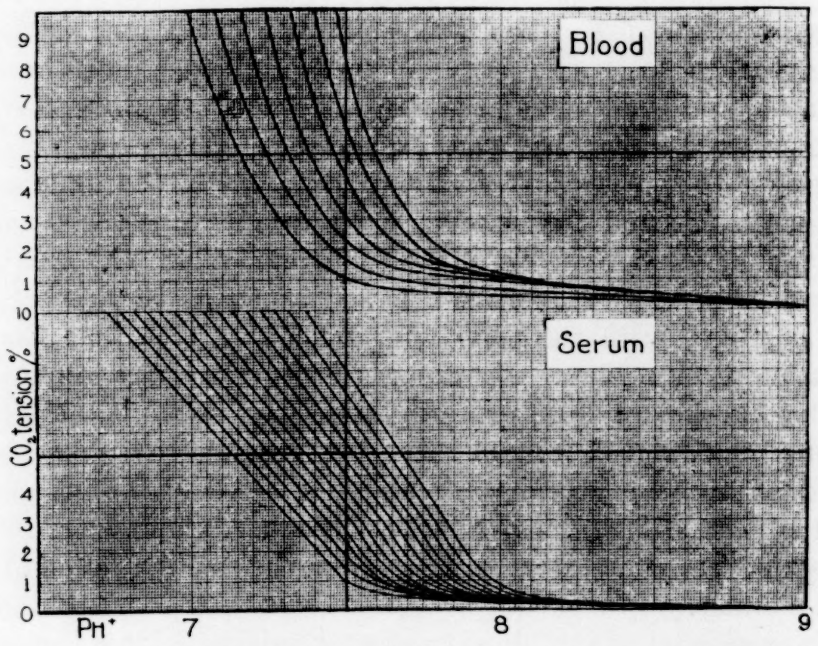

FIG. 3. Curves showing the change of P_H (= − log H ion concentration) with change of CO_2 tension. On the ordinates are measured the percentages of an atmosphere in moist CO_2, and these may be reduced to mm. of dry CO_2 by multiplying by 7.2, since the tension of water vapor was 20 mm. and the barometric pressure 740 mm. At high altitudes all calculations should be made in mm., but near the sea level the error is not appreciable if the calculations are made in percentage of an atmosphere, as a slight change in CO_2 tension makes a much smaller change in P_H. The electrode potential calculated for 760 mm. of dry hydrogen was used in determining the P_H.

Since the P_H of arterial blood both in health and disease is remarkably constant (about 7.5) the curve in the chart corresponding to any sample of blood or serum may be found by one observation. If the alveolar CO_2 tension by the Haldane method is determined, the curve is the one passing through the intersection of this CO_2 tension and the $P_H = 7.5$ line. If the P_H at any known CO_2 tension is determined, the curve is the one

at the intersection of these P_H and CO_2 tension coordinates, and the intersection of this curve with the $P_H = 7.5$ line shows the alveolar CO_2 tension (unless the respiratory center is abnormal as in shock or after caffeine or morphine).

The alveolar CO_2 tension by Haldane's method is practically the same as that of the arterial blood. The alveolar CO_2 tension by Plesch's method may, by proper manipulation, be made to show the average CO_2 tension of the venous blood. The normal arterial CO_2 tension of the arterial blood is about 5.2 per cent, and of the average venous blood about 6 per cent of an atmosphere, or about 16 per cent higher than arterial. Higgins used a modified Plesch apparatus, in which the entire air was rebreathed four times (about 20 seconds), and obtained values about 20 per cent higher than by Haldane's method. It would be interesting to know whether such a ratio is constant even when cases of extremely low CO_2 tension are included. If such were the case, the true alveolar CO_2 tension might be calculated from the results by the Plesch method.

The striking fact shown by the charts is that the buffer value of the blood or serum depends on its CO_2 tension. Below 1 per cent CO_2 tension, the buffer value is almost nil. Above 5 per cent CO_2 tension, the buffer value of the blood is almost infinite, while that of the serum is somewhat less. Therefore, the buffer value of the blood of a patient with alveolar CO_2 tension equal to less than 1 per cent of an atmosphere, is so low as to make it probable that great local differences in the reaction of the blood exist in the body. Such local variations probably have a great deal to do with the dyspnea of many patients. The acid produced in the respiratory center itself may be very poorly neutralized by the blood, and temporary increased breathing must result.

5. The Indicator Method for Determining the Buffer Value.

At the request of Dr. Rowntree we have calibrated his indicator method for determining the reaction of blood. Phosphates of known water content were used to make solutions according to Sörensen's directions, and portions of the same phosphates sent to Hynson, Westcott and Company for the manufacture of

sealed tubes of standard solutions colored with phenolsulfonephthalein. These tubes were then compared with similar ones calibrated by the gas chain method. The only difficulty experienced was in obtaining "nonsol" tubes of exactly 1 cm. bore, a small divergence from which caused an appreciable error. Blood was tested by the gas chain method and then dialyzed 7 minutes against isotonic, neutral (tested) NaCl solution in *stoppered* "nonsol" tubes and tested with phenolsulfonephthalein. If the transfer and other manipulations were sufficiently rapid so as not to lose CO_2 appreciably into the air, and the tubes were of exactly 1 cm. bore, the results did not disagree with those of the gas chain method. The latter could be read, however, to one more decimal place. In attempting to prevent CO_2 loss by a layer of oil, it should be remembered that CO_2 is more soluble in oil than in water, and the oil merely lessens convection (by its non-miscibility).

The following method for determining the buffer value clinically is suggested. 3 or more cc. of blood mixed with hirudin or neutralized oxalate are introduced into a 100 cc. tube with openings at each end of 1 cm. bore provided with rubber tubes and pinch-cocks. The blood is introduced into this tube and the breath of a person of normal (previously tested) alveolar CO_2 tension blown through it, the pinch-cocks being closed after the last breath is forcibly expelled. The tube is rotated 10 minutes and the reaction of the blood determined by the indicator-dialysis method. The intersection of the line of this P_H with the 5.2 per cent CO_2 tension line in the chart will show the curve corresponding to the blood sample. The intersection of this curve with the 7.5 P_H line will show the alveolar CO_2 tension of the patient (the error will be small except in case of shock or after certain drugs).

6. The Indicator Method for P_H of Stomach Contents.

Stomach contents were dialyzed through collodion sacs as first tried by Fowler, Bergeim, and Hawk. The buffer value varies directly with the P_H and is very low for high acidity, in which case the volume of the stomach contents in the sac should far exceed that of the 1 per cent NaCl solution in the tube containing the sac. For low acidity, Davidsohn has shown that dilution affects the P_H very little.

The dialysate was compared with the published colored chart (McClendon, 1916 b), using the indicators noted in the chart. A calibration of this method showed that it was significant for the integral part of the P_H. Sealed tubes of standard solutions with some of the indicators were made also but, unfortunately, methyl violet, the most useful indicator, fades in very acid solutions. Mauveine was not obtainable, but it is hoped that it would not fade so rapidly. Neutral red shows a striking change to blue in very acid solutions, but the acidity required is higher than is usually the case with stomach contents.

In conclusion, thanks are due to Dr. J. W. Northington and Miss Swift for aid in the collection of blood samples.

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INDEX TO VOLUME XXV.

AUTHORS.

A

Alsberg, Carl L., and **Black, Otis F.** The separation of autogenous and added hydrocyanic acid from certain plant tissues and its disappearance during maceration, 133

—. See VIEHOEVER, JOHNS, and ALSBERG, 141

Anderson, R. J. The utilization of inosite in the dog, 391

—, and **Bosworth, A. W.** The utilization of inosite in the animal organism. The effect of inosite upon the metabolism of man, 399

Aschner, Paul W. See EPSTEIN and ASCHNER, 151

B

Baker, Willis. See GETTLER and BAKER, 211

Baumann, Louis, and **Ingvaldsen, Thorsten.** The determination of creatine in muscle. III, 195

Black, Otis F. See ALSBERG and BLACK, 133

Bloor, W. R. The distribution of the lipoids (fat) in human blood, 577

Bosworth, A. W. See ANDERSON and BOSWORTH, 399

Bradley, H. C. Is autolysis an autocatalytic phenomenon? An interpretation, 201

—, and **Taylor, Joseph.** Studies of autolysis. III. The effect of reaction on liver autolysis, 261

—, and —. Studies of autolysis. IV. The latent period in autolysis, 363

C

Clark, William Mansfield, and **Lubs, Herbert A.** Hydrogen electrode potentials of phthalate, phosphate, and borate buffer mixtures, 479

Coffin, H. W. See KRAMER and COFFIN, 423

Collins, R. J., and **Hanzlik, P. J.** A colorimetric method for the estimation of free formaldehyde and hexamethyleneamine, 231

Cutler, Ethel. See ROBERTSON and CUTLER, 663

E

Epstein, Albert A., and **Aschner, Paul W.** The effect of surgical procedures on the blood sugar content, 151

Ewald, W. F. See LOEB and EWALD, 377

F

Falls, Frederick H. See WELKER and FALLS, 567

Fenger, Frederic. The composition and physiological activity of the pituitary body. II, 417

Ferry, Edna L. See OSBORNE and MENDEL, 1
Funk, Casimir. The nature of the disease due to the exclusive diet of oats in guinea pigs and rabbits, 409

G

Germann, Hildegarde C. See JONES and GERMANN, 93
—. The partition of phosphorus in thymus nucleic acid, 189
Gettler, A. O., and **Baker, Willis.** Chemical and physical analysis of blood in thirty normal cases, 211
Green, Helen S. See RICHARDSON and GREEN, 307
Greenwald, Isidor. The fate of normal α-aminocaproic acid in the phlorhizinized dog, 81
—. Some observations on the tetany of parathyroidectomized dogs, 223
—. The nature of the acid-soluble phosphorus of serum, 431

H

Hammett, Frederick S. The effect on nitrogen partition of substituting alcohol for sucrose in an otherwise fixed diet, 601
Hanzlik, P. J. See COLLINS and HANZLIK, 231
Harding, Victor John, and **Warneford, Francis H. S.** The ninhydrin reaction with amino-acids and ammonium salts, 319
—, and **MacLean, Reginald M.** The ninhydrin reaction with amines and amides, 337
Hart, E. B., Miller, W. S., and **McCollum, E. V.** Further studies on the nutritive deficiencies of wheat and grain mixtures and the pathological conditions produced in swine by their use, 239
Holmes, August. See JACOBSON and HOLMES, 29
—. See JACOBSON and HOLMES, 55
Hulton, Florence. The formation of specific proteoclastic ferments in response to the parenteral injection of foreign proteins, 163
—. See TAYLOR, A. E., and HULTON, 173
—. The formation of specific proteoclastic ferments in response to introduction of placenta, 227

I

Ingvaldsen, Thorsten. See BAUMANN and INGVALDSEN, 195

J

Jacobs, W. A. See LEVENE and JACOBS, 103
Jacobson, C. A., and **Holmes, August.** Solubility data for various salts of lauric, myristic, palmitic, and stearic acids, 29
—, and —. The separation of lauric and myristic acids from each other and from mixtures of other fatty acids, 55
Janney, N. W. The quantitative determination of the total protein and non-protein substances of muscle. Improved technique, 177

—. The protein content of muscle, 185
Jewett, R. M. Studies in the blood relationship of animals as displayed in the composition of the serum proteins. V. The percentage of non-proteins in the sera of certain animals and birds, 21
Johns, Carl O. See VIEHOEVER, JOHNS, and ALSBERG, 141
Jones, Walter. The admissibility of ammonium magnesium phosphate as a form in which to weigh phosphoric acid, 87
—, and **Germann, Hildegarde C.** Hydrolysis of yeast nucleic acid with ammonia, 93

K

Karr, Walter G. See LEWIS and KARR, 13
Kocher, R. A. The mechanism of the sparing action of carbohydrates on protein metabolism, 571
Kramer, B., and **Coffin, H. W.** The rôle of psychic and sensory stimuli in the hyperglycemia produced by lowering the environmental temperature of dogs, 423
Kuriyama, Shigenobu. The utilization of sucrose and the inverting power of the blood serum after parenteral administration of sucrose, 521

L

Levene, P. A., and **Jacobs, W. A.** Note on hydrolysis of yeast nucleic acid in the autoclave, 103
—, and **López-Suárez, J.** The conjugated sulfuric acid of the mucin of pig's stomach (mucoitin sulfuric acid), 511
—, and **Senior, James K.** Vicine and divicine, 607
—, and —. The preparation of guanidine sulfate, 623
—, and **West, C. J.** Cephalin. IV. Phenyl- and naphthylureidocephalin, 517
Lewis, Howard B., and **Karr, Walter G.** Studies in the synthesis of hippuric acid in the animal organism. III. The excretion of uric acid in man after ingestion of sodium benzoate, 13
Loeb, Jacques, and **Ewald, W. F.** Chemical stimulation of nerves, 377
López-Suárez, J. See LEVENE and LÓPEZ-SUÁREZ, 511
Lubs, Herbert A. See CLARK and LUBS, 479

M

McClendon, J. F., and **Magoon, C. A.** An improved Hasselbalch hydrogen electrode and a combined tonometer and hydrogen electrode, together with rapid methods of determining the buffer value of blood, 669
McCollum, E. V., Simmonds, Nina, and **Pitz, Walter.** The nature of the dietary deficiencies of the wheat embryo, 105
—. See HART, MILLER, and MCCOLLUM, 239
MacLean, Reginald, M. See HARDING and MACLEAN, 337
Magoon, C. A. See MCCLENDON and MAGOON, 669

Marshall, E. K., Jr. See MATEER and MARSHALL, 297
Mateer, J. G., and **Marshall, E. K., Jr.** The urease content of certain beans, with special reference to the jack bean, 297
Mendel, Lafayette B. See OSBORNE and MENDEL, 1
Miller, C. W. See TAYLOR, A. E., and MILLER, 281
Miller, W. S. See HART, MILLER, and MCCOLLUM, 239
Miyake, K. The toxic action of soluble aluminium salts upon the growth of the rice plant, 23
—. See ROBERTSON and MIYAKE, 351
Morris, J. Lucien. A new salt of uric acid and its application to the analysis of uric acid and phenol, 205
Mueller, J. Howard. A comparison of the results obtained by the colorimetric and gravimetric determinations of cholesterol, 549
—. The influence of autolysis upon cholesterol esters, 561

O

Osborne, **Thomas B.,** and **Mendel, Lafayette B.** The amino-acid minimum for maintenance and growth, as exemplified by further experiments with lysine and tryptophane, 1

P

Pitz, Walter. See MCCOLLUM, SIMMONDS, and PITZ, 105

R

Richardson, Anna E., and **Green, Helen S.** Nutrition investigations upon cottonseed meal. I, 307
Robertson, T. Brailsford, and **Miyake, K.** The influence of alkali and alkaline earth salts upon the rate of solution of casein by sodium hydroxide, 351
—. Experimental studies on growth. V. The influence of cholesterol upon the growth of the white mouse, 635
—. Experimental studies on growth. VI. The influence of lecithin upon the growth of the white mouse, 647
—, and **Cutler, Ethel.** Experimental studies on growth. VII. The influence of the administration of egg lecithin and of cholesterol to the mother, upon the growth of suckling mice, 663

S

Schmidt, Carl L. A. Changes in the H^+ and OH^- concentration which take place in the formation of certain protein compounds, 63
Senior, James K. See LEVENE and SENIOR, 607
—. See LEVENE and SENIOR, 623
Simmonds, Nina. See MCCOLLUM, SIMMONDS, and PITZ, 105

T

Taylor, A. E., and **Hulton, Florence.** The limit of assimilation of glucose, 173

—, and **Miller, C. W.** Studies in Bence-Jones proteinuria, 281

Taylor, Joseph. See BRADLEY and TAYLOR, 261

—. See BRADLEY and TAYLOR, 363

U

Underhill, Frank P. Studies in carbohydrate metabolism. XI. The rôle of calcium in the regulation of blood sugar content, 447

—. Studies in carbohydrate metabolism. XII. The influence of sodium carbonate upon blood sugar content and upon epinephrine hyperglycemia and glycosuria, 463

—. Studies in carbohydrate metabolism. XIII. The influence of magnesium salts upon blood sugar content and upon epinephrine hyperglycemia and glycosuria, 471

V

Viehoever, Arno, Johns, Carl O., and **Alsberg, Carl L.** Cyanogenesis in plants. Studies on *Tridens flavus* (tall red top), 141

W

Wakeman, Alfred J. See OSBORNE and MENDEL, 1

Warneford, Francis H. S. See HARDING and WARNEFORD, 319

Welker, William H., and **Falls, Frederick H.** Studies on blood serum. I. The determination of non-colloidal nitrogen, 567

West, C. J. See LEVENE and WEST, 517

Williams, Robert R. The chemical nature of the vitamines. I. Antineuritic properties of the hydroxypyridines, 437

Wulzen, Rosalind. The pituitary gland. Its effect on growth and fission of planarian worms, 625

SUBJECTS.

A

Abderhalden reaction:
(HULTON), 163, 227

Acetone:
α-Aminocaproic acid, formation from (GREENWALD), 82
Fatty acid salts, solubility of, in (JACOBSON and HOLMES), 38

Acid:
Bence-Jones urine, behavior of, towards (TAYLOR and MILLER), 285
Latent period in liver autolysis, effect on (BRADLEY and TAYLOR), 364
Liver autolysis, effect on (BRADLEY and TAYLOR), 262
-Soluble phosphorus of serum (GREENWALD), 431

Acid-base equilibria:
Blood sugar regulation, rôle in (UNDERHILL), 466

Acidosis:
Oat diet, production by, in rabbits (FUNK), 410
Protein relationships in (BRADLEY and TAYLOR), 277

Adenine-uracil dinucleotide:
Yeast nucleic acid, preparation from (JONES and GERMANN), 100

Adrenalin:
Ninhydrin reaction with (HARDING and MACLEAN), 343

Albumin:
Milk, serum, normal and sensitized, digestion by (HULTON), 168, 228
Sera of animals and birds, content of (JEWETT), 21

Alcohol:
Nitrogen partition, effect on, when substituted for sucrose (HAMMETT), 601
Protein, precipitation of (JANNEY), 178

Alfalfa:
Growth, influence on (HART, MILLER, and MCCOLLUM), 246

Alkali:
Blood reserve (GETTLER and BAKER), 219
Liver autolysis, effect on (BRADLEY and TAYLOR), 268
Salts, casein, rate of solution of, in sodium hydroxide, effect on (ROBERTSON and MIYAKE), 351

Alkaline earth salts:
Casein, rate of solution of, in sodium hydroxide, effect on (ROBERTSON and MIYAKE), 351

Alloxan:
Murexide formation from, ninhydrin reaction, analogy to (HARDING and WARNEFORD), 320
Triketohydrindene hydrate (ninhydrin), relation to (HARDING and MACLEAN), 334
Aluminium chloride:
Hydrogen ion concentration of solutions of (MIYAKE), 26
Rice plant, toxic action on growth of (MIYAKE), 23
Aluminium hydroxide cream:
Serum colloids, precipitation of (WELKER and FALLS), 567
Amides:
Ninhydrin reaction with (HARDING and MACLEAN), 337
Amines:
Ninhydrin reaction with (HARDING and MACLEAN), 337
Amino-acid:
Autolysis, rapid, lag in (BRADLEY and TAYLOR), 367
Growth, minimum for (OSBORNE and MENDEL), 1
Liver autolysis, latent period in (BRADLEY and TAYLOR), 364
Maintenance, minimum for (OSBORNE and MENDEL), 1
Ninhydrin reaction with (HARDING and WARNEFORD), 319
α-Aminocaproic acid:
Fate in phlorhizinized dog (GREENWALD), 81
α-Aminocaproic acid–*continued:*
Glucose from (GREENWALD), 81
Ammonia:
Blood content (GETTLER and BAKER), 215
Inosite, effect of, on excretion of (ANDERSON and BOSWORTH), 404
Ninhydrin reaction with (HARDING and WARNEFORD), 330
Yeast nucleic acid, hydrolysis of, with (JONES and GERMANN), 73
Ammonium magnesium phosphate:
Composition (JONES), 90
(GREENWALD), 433
Phosphoric acid weighed as (JONES), 87
Ammonium salts:
Ninhydrin reaction with (HARDING and WARNEFORD), 319
— — —, pyridine, influence of (HARDING and WARNEFORD), 324
Amygdalin:
Tridens flavus, enzymatic action of (VIEHOEVER, JOHNS, and ALSBERG), 149
Amyl acetate:
Fatty acid salts, solubility of, in (JACOBSON and HOLMES), 37
Amyl alcohol:
Fatty acid salts, solubility of, in (JACOBSON and HOLMES), 36
Anaphylaxis:
Bence-Jones protein (TAYLOR and MILLER), 290

Andropogon sorghum:
Hydrocyanic acid content of leaves (ALSBERG and BLACK), 136
Anesthesia:
Blood sugar, effect on (EPSTEIN and ASCHNER), 156
Hyperglycemia, production of (EPSTEIN and ASCHNER), 152
Magnesium sulfate, hydrochloric acid, influence of (UNDERHILL), 477
— —, sodium carbonate, influence of (UNDERHILL), 477
Antiscorbutics:
Oat disease in rabbits, effect on (FUNK), 412
Apparatus:
Hydrogen electrode, improved Hasselbalch (McCLENDON and MAGOON), 669
Tonometer and hydrogen electrode (McCLENDON and MAGOON), 675
Assimilation:
Glucose, limit of (TAYLOR and HULTON), 173
Atrophy:
Protein relationships in (BRADLEY and TAYLOR), 279
Autolysis:
Amino-acid lag in rapid (BRADLEY and TAYLOR), 367
Autocatalytic phenomenon (BRADLEY), 201
Cholesterol esters, effect on (MUELLER), 561
Autolysis—*continued:*
Latent period in (BRADLEY and TAYLOR), 363
Liver, effect of reaction on (BRADLEY and TAYLOR), 261
Substratum, rôle of, in (BRADLEY and TAYLOR), 368

B

Barium chloride:
Casein, rate of solution of, in sodium hydroxide, influence on (ROBERTSON and MIYAKE), 357
Barium salts:
Fatty acids, solubility of, in organic solvents (JACOBSON and HOLMES), 45
Bases:
Ninhydrin reaction with (HARDING and MACLEAN), 337
Bence-Jones:
Protein. *See* Protein.
Urine. *See* Urine.
Benzene:
Fatty acid salts, solubility of, in (JACOBSON and HOLMES), 50
Benzoic acid:
Creatinine excretion, influence on (LEWIS and KARR), 16
Excretion following sodium benzoate or hippurate (LEWIS and KARR), 20
Uric acid excretion, influence on (LEWIS and KARR), 16

Beryllium salts:
Fatty acids, solubility of, in organic solvents (JACOBSON and HOLMES), 32

Blood:
Alkali reserve (GETTLER and BAKER), 219
Ammonia content (GETTLER and BAKER), 215
Autolyzed, cholesterol content (MUELLER), 565
Buffer value of (McCLENDON and MAGOON), 677
— — —, charts for (McCLENDON and MAGOON), 678
— — —, indicator method for (McCLENDON and MAGOON), 679
Carbon dioxide tension, buffer value, relation to (McCLENDON and MAGOON), 679
Chloride content (GETTLER and BAKER), 219
Cholesterol content (GETTLER and BAKER), 218
(BLOOR), 585
—, determination of, (GETTLER and BAKER), 218
(MUELLER), 554
Circulating, conductivity of (GETTLER and BAKER), 219
Creatine content (GETTLER and BAKER), 216
Creatinine content (GETTLER and BAKER), 216

Blood—*continued:*
Defibrinated, hydrogen ion concentration (McCLENDON and MAGOON) 672
Fat content (GETTLER and BAKER), 218
(BLOOR), 585
—, determination of (GETTLER and BAKER), 218
Freezing point (GETTLER and BAKER), 221
Human, lipoids, distribution of (BLOOR), 577
Hydrogen ion concentration of (GETTLER and BAKER), 221
(McCLENDON and MAGOON), 672
Hyperglycemia produced by loss of (EPSTEIN and ASCHNER), 162
Intestinal sucrase activity, effect on (KURIYAMA), 541
Invertin activity, effect on (KURIYAMA), 541
Inverting power after parenteral injection of sucrose (KURIYAMA), 534
Lecithin content (BLOOR), 585
Nitrogen content (GETTLER and BAKER), 213
Non-protein nitrogen content (GETTLER and BAKER), 213
— — —, determination of (GETTLER and BAKER), 214
Normal, chemical and physical analysis (GETTLER and BAKER), 211

Blood—*continued:*
Proteoclastic ferments, formation of, in (HULTON), 167, 227
Refractive index (GETTLER and BAKER), 221
Relationships of animals (JEWETT), 21
Serum, invertin content after injection of invertin (KURIYAMA), 539
—, inverting power of, after parenteral administration of sucrose (KURIYAMA), 534
—, nitrogen, non-colloidal determination of (WELKER and FALLS), 567
Solids, content of (GETTLER and BAKER), 213
Specific gravity (GETTLER and BAKER), 221
Sugar content (TAYLOR and HULTON), 174
(GETTLER and BAKER), 217
— —, before anesthesia (EPSTEIN and ASCHNER), 152
— —, anesthesia, effect of (EPSTEIN and ASCHNER), 156
— —, calcium chloride, effect of (UNDERHILL), 449
— —, calcium lactate, effect of (UNDERHILL), 449
— —, cold, effect of (KRAMER and COFFIN), 426

Blood—*continued:*
— —, epinephrine, effect of (UNDERHILL), 450
— —, glucose, effect of large amounts of (TAYLOR and HULTON), 173
— —, local anesthesia, effect of (EPSTEIN and ASCHNER), 156
— —, magnesium lactate, effect of (UNDERHILL), 472
— —, magnesium sulfate, effect of (UNDERHILL), 474
— —, nitrous oxide anesthesia, effect of (EPSTEIN and ASCHNER), 157
— —, — — and ether anesthesia, effect of (EPSTEIN and ASCHNER), 157
— —, psychic factors, effect of (EPSTEIN and ASCHNER), 154
— —, sodium carbonate, effect of (UNDERHILL), 463
— —, — oxalate, effect of (UNDERHILL), 456
— —, — phosphate, effect of (UNDERHILL), 456
— —, surgical procedures, effect of (EPSTEIN and ASCHNER), 151
Urea content (GETTLER and BAKER), 215
Uric acid content (GETTLER and BAKER), 215
Venous, hydrogen ion concentration (McCLENDON and MAGOON), 674

Borate buffer mixtures:
Hydrogen electrode potential of (CLARK and LUBS), 479

Buffer value:
Blood, carbon dioxide tension, relation of (McCLENDON and MAGOON), 679
—, charts for (McCLENDON and MAGOON), 678
—, clinical determination of (McCLENDON and MAGOON), 680
Indicator method for (McCLENDON and MAGOON), 679
Serum, carbon dioxide tension, relation to (McCLENDON and MAGOON), 679
—, charts for (McCLENDON and MAGOON), 678

Butter fat:
Growth, influence on (McCOLLUM, SIMMONDS, and PITZ), 109

C

Calcium:
Blood sugar content, rôle in regulation of (UNDERHILL), 447
Growth in swine, effect on (HART, MILLER, and McCOLLUM), 247

Calcium carbonate:
Liver autolysis, effect on (BRADLEY and TAYLOR), 266

Calcium chloride:
Blood sugar content, effect on (UNDERHILL), 449

Calcium chloride—*continued:*
Casein, rate of solution of, in sodium hydroxide, effect on (ROBERTSON and MIYAKE), 355
Glycosuria epinephrine, effect on (UNDERHILL), 451
Hyperglycemia, epinephrine, effect on (UNDERHILL), 451
Nerve stimulation, inhibitory effect on (LOEB and EWALD), 380

Calcium lactate:
Blood sugar, influence on (UNDERHILL), 449
Glycosuria, epinephrine, effect on (UNDERHILL), 451
Hyperglycemia, epinephrine, effect on (UNDERHILL), 451

Calomel:
Electrodes, preparation of (CLARK and LUBS), 484
—, tenth normal, value of (CLARK and LUBS), 494

Canavalia ensiformis:
Urease content (MATEER and MARSHALL), 298

Canavalia gladiata:
Urease content (MATEER and MARSHALL), 298

Carbohydrate:
Metabolism (UNDERHILL), 447, 463, 471
Protein metabolism, sparing action on (KOCHER), 571

Carbon dioxide:
Liver autolysis, effect on (BRADLEY and TAYLOR), 262
Plasma, capacity of (GETTLER and BAKER), 219
Tension, blood buffer value, relation to (McCLENDON and MAGOON), 679
—, serum buffer value, relation to (McCLENDON and MAGOON), 679
Casein:
Globin, compound of (SCHMIDT), 73
Liver enzymes, digestion by (BRADLEY and TAYLOR), 269
Serum, digestion by normal and sensitized (HULTON), 168, 228
Solution by sodium hydroxide, alkali and alkaline earth salts, influence of, on rate of (ROBERTSON and MIYAKE), 351
Catabolism:
α-Aminocaproic acid (GREENWALD), 81
Cation:
Nerve stimulation, rôle in (LOEB and EWALD), 379
Cell:
Conductivity (GETTLER and BAKER), 219
Liver, protein relationships in (BRADLEY and TAYLOR), 276
Cephalin:
Ureido derivatives (LEVENE and WEST), 517
Chemical:
Blood, analyses of normal (GETTLER and BAKER), 211
Chlorides:
Blood content (GETTLER and BAKER), 210
Chloroform:
Fatty acid salts, solubility of, in (JACOBSON and HOLMES), 36
Cholesterol:
Blood content (GETTLER and BAKER), 218
(BLOOR), 585
—, determination in (GETTLER and BAKER), 218
(MUELLER), 554
Determination, colorimetric and gravimetric methods compared (MUELLER), 549
Growth of suckling mice, influence on, when fed to mothers (ROBERTSON and CUTLER), 663
— of white mice, influence on (ROBERTSON), 635
Cholesterol esters:
Autolysis, effect of (MUELLER), 561
Circulation:
Sucrose, inversion of, by, invertin in the (KURIYAMA), 524
Cold:
Hyperglycemia produced by, effect of psychic and sensory stimuli (KRAMER and COFFIN), 423
Colorimetric methods:
Hydrogen ion concentration, comparison solutions for (CLARK and LUBS), 479

Concentration:
Coefficient of penetration, relation of, to (ROBERTSON and MIYAKE), 359
Conductivity:
Blood, circulating (GETTLER and BAKER), 219
Cell (GETTLER and BAKER), 219
Serum (GETTLER and BAKER), 220
Congo red:
Standardization of (COLLINS and HANZLIK), 232
Copper carbonate:
Liver autolysis, effect on (BRADLEY and TAYLOR), 266
Corn oil:
Nutritive value (McCOLLUM, SIMMONDS, and PITZ), 111
Cottonseed:
Flour, growth, influence on (RICHARDSON and GREEN), 310
Meal, milk powder and, growth on (RICHARDSON and GREEN), 313
—, nutrition investigations (RICHARDSON and GREEN), 307
—, nutritive factors absent in (RICHARDSON and GREEN), 312
—, toxicity (RICHARDSON and GREEN), 314
Creatine:
Blood content (GETTLER and BAKER), 216
Creatinine, conversion into (BAUMANN and INGVALDSEN), 197
Creatine—*continued:*
Muscle, determination in (BAUMANN and INGVALDSEN), 195
Creatinine:
Blood content (GETTLER and BAKER), 216
Creatine, conversion of, into (BAUMANN and INGVALDSEN), 197
Excretion, sodium benzoate, effect of (LEWIS and KARR), 16
—, — hippurate, effect of (LEWIS and KARR), 20
Inosite, effect of, on excretion of (ANDERSON and BOSWORTH), 404
Cyanogenesis:
(ALSBERG and BLACK), 133
(VIEHOEVER, JOHNS, and ALSBERG), 141

D

Deuteroalbumose:
Globin, compound with (SCHMIDT), 78
Dextrose:
Serum, action of (KURIYAMA), 538
Dialuric acid:
Ninhydrin reaction with ammonium salts, action on (HARDING and WARNEFORD), 330
2, 5-Diamino-4, 6-dioxypyrimidine sulfate:
(LEVENE and SENIOR), 614
Potassium chlorate, action of (LEVENE and SENIOR), 615
Urea, action of (LEVENE and SENIOR), 616

4, 5-Diamino-2, 6-dioxypyrimidine sulfate:
(Levene and Senior), 613
Urea, action of (Levene and Senior), 616
Dicyandiamide:
Guanidine from (Levene and Senior), 623
Diffusion:
Potentials (Clark and Lubs), 483
Digestibility:
Bence-Jones protein (Taylor and Miller), 293
Digitonin:
Cholesterol, determination of, by (Mueller), 549
Diketohydrindylidene-diketohydrindamine:
Ammonium salt, detection of (Harding and Warneford), 321
— —, murexide, relation to (Harding and MacLean), 346
Dipotassium hydrogen phosphate:
Liver autolysis, effect on (Bradley and Taylor), 265
Disease:
Oat, nature of (Funk), 409
Divicine:
Constitution (Levene and Senior), 607
Sulfate (Levene and Senior), 614
—, potassium chlorate, action of (Levene and Senior), 615
—, urea, action of (Levene and Senior), 616
Dolichos biflorus:
Urease content (Mateer and Marshall), 298

E

Edestin:
Serum, normal and sensitized, digestion by (Hulton), 168, 228
Egg:
Lecithin. *See* Lecithin.
Electrodes:
Calomel, preparation of (Clark and Lubs), 484
Hydrogen, improved Hasselbalch (McClendon and Magoon), 669
—, normal (Clark and Lubs), 494
Palladium black (Clark and Lubs), 485
Embryo:
Wheat. *See* Wheat.
Emulsin:
Tridens flavus, action on (Viehoever, Johns, and Alsberg), 145
Enzymes:
Tridens flavus (Viehoever, Johns, and Alsberg), 144
Epinephrine:
Blood sugar content, influence on (Underhill), 450
Glycosuria. *See* Glycosuria.
Hyperglycemia. *See* Hyperglycemia.
Urine sugar content, influence on (Underhill), 450

Ether:
Fatty acid salts, solubility of, in (JACOBSON and HOLMES), 35
Nitrous oxide and, anesthesia, blood sugar content, effect on (EPSTEIN and ASCHNER), 157
Ethyl acetate:
Fatty acid salts, solubility of, in (JACOBSON and HOLMES), 50
Ethyl alcohol:
Fatty acid salts, solubility of, in (JACOBSON and HOLMES), 33
Ethylene glycol:
Ninhydrin reaction with ammonium salts, action on (HARDING and WARNEFORD), 330

F

Factor:
Protein (JANNEY), 185
Fat:
Blood content (GETTLER and BAKER), 218
(BLOOR), 585
—, determination in (GETTLER and BAKER), 218
Fatty acids:
Separation of, by solubility of their salts (JACOBSON and HOLMES), 55
Feces:
Inosite, excretion of, in (ANDERSON), 395
Ferments:
Proteoclastic, specific, formation of, in response to introduction of placenta (HULTON), 227
Ferments—*continued:*
—, —, — —, — — — —
— proteins (HULTON), 163
Ferric hydroxide:
Liver autolysis, effect on (BRADLEY and TAYLOR), 267
Fission:
Planarian worms, pituitary body, effect of, on (WULZEN), 625
Formaldehyde:
Determination of, colorimetric (COLLINS and HANZLIK), 231
Freezing point:
Blood (GETTLER and BAKER), 221

G

Gelatin:
Latent period in liver autolysis, effect on (BRADLEY and TAYLOR), 368
Liver enzymes, digestion by (BRADLEY and TAYLOR), 272
Gliadin:
Feeding experiments with (OSBORNE and MENDEL), 5
Serum, normal and sensitized, digestion by (HULTON), 168, 228
Globin:
Casein, compound with (SCHMIDT), 73
Deuteroalbumose, compound with (SCHMIDT), 78
Nucleic acid, compound with (SCHMIDT), 76

Globin—*continued:*
Salting out of, changes in H^+ and OH^- concentrations during (SCHMIDT), 69
Taurocholic acid, compound with (SCHMIDT), 76

Globulin:
Sera of animals and birds, content of (JEWETT), 21
Soy bean, serum, normal and sensitized, digestion by (HULTON), 168, 228

Glucosamine:
Mucoitin sulfuric acid, presence in (LEVENE and LÓPEZ-SUÁREZ), 514

Glucose:
α-Aminocaproic acid, formation from, in phlorhizinized dog (GREENWALD), 81
Assimilation, limit of (TAYLOR and HULTON), 173
Ingestion, limit of (TAYLOR and HULTON), 175
Ninhydrin reaction with ammonium salts, action on (HARDING and WARNEFORD), 330

Glycerol:
Ninhydrin reaction with ammonium salts, action on (HARDING and WARNEFORD), 330

Glycine hispida:
Urease content (MATEER and MARSHALL), 298

Glycosuria:
Epinephrine, calcium chloride and lactate, influence on (UNDERHILL), 451
—, magnesium lactate and sulfate, influence of (UNDERHILL), 472
—, sodium carbonate, influence of (UNDERHILL), 467
—, sodium oxalate and phosphate, influence of (UNDERHILL), 456
Magnesium sulfate, calcium chloride, effect of (UNDERHILL), 475
Post-operative (EPSTEIN and ASCHNER), 162

Glyoxals:
Ninhydrin reaction, intermediate products of (HARDING and WARNEFORD), 327

Grain:
Nutritive deficiencies of mixtures of (HART, MILLER, and MCCOLLUM), 239

Grasses:
Hydrocyanic acid content (VIEHOEVER, JOHNS, and ALSBERG), 141

Growth:
Alfalfa meal, influence of (HART, MILLER, and MCCOLLUM), 246
Amino-acid minimum for (OSBORNE and MENDEL), 1
Chicks, pituitary body, effect of (WULZEN), 630

Growth—*continued:*
Cholesterol, influence of, on white mouse (ROBERTSON), 635
—, — —, on suckling mice, when fed to mother (ROBERTSON and CUTLER), 663
Cottonseed meal and milk powder, influence of (RICHARDSON and GREEN), 313
Curves of (OSBORNE and MENDEL), 9
(MCCOLLUM, SIMMONDS, and PITZ), 115
(HART, MILLER, and MCCOLLUM), 250
(RICHARDSON and GREEN), 316
Lecithin, influence of, on mice (ROBERTSON), 647
—, — —, on suckling mice, when fed to mother (ROBERTSON and CUTLER), 663
Lysine, rôle of (OSBORNE and MENDEL), 1
Meat scraps, effect of (HART, MILLER, and MCCOLLUM), 247
Planarian worms, pituitary body, effect of (WULZEN), 625
Rice plant, hydrochloric acid, influence of (MIYAKE), 26
— —, toxic action of aluminium salts (MIYAKE), 23
Salts, influence of (HART, MILLER, and MCCOLLUM), 245
Tryptophane, rôle in (OSBORNE and MENDEL), 1
Growth—*continued:*
Wheat embryo, value of, for (MCCOLLUM, SIMMONDS, and PITZ), 107
Guanidine:
Ninhydrin reaction negative (HARDING and MACLEAN), 348
Sulfate, preparation (LEVENE and SENIOR), 623
Guanylic acid:
Triphosphonucleinsäure, identity with (JONES and GERMANN), 99

H

Heat:
Bence-Jones protein, reaction of (TAYLOR and MILLER), 282
Hemoglobin:
Liver enzymes, digestion by (BRADLEY and TAYLOR), 273
Hexamethyleneamine:
See Hexamethylenetetramine.
Hexamethylenetetramine:
Determination, colorimetric (COLLINS and HANZLIK), 231
Hippuric acid:
Creatinine excretion, influence on (LEWIS and KARR), 20
Synthesis in animal organism (LEWIS and KARR), 13
Uric acid determination in urine, effect on (LEWIS and KARR), 14
— — excretion, effect on (LEWIS and KARR), 19

Horse gram:
Urease content (MATEER and MARSHALL), 298
Hydrochloric acid:
Magnesium sulfate anesthesia, influence on (UNDERHILL), 477
Rice plant, influence on growth of (MIYAKE), 26
Hydrocyanic acid:
Liver autolysis, effect on (BRADLEY and TAYLOR), 262
Plant tissues, disappearance from, during maceration (ALSBERG and BLACK), 133
— —, separation from (ALSBERG and BLACK), 133
Tridens flavus, content of (VIEHOEVER, JOHNS, and ALSBERG), 141
Hydrogen:
Electrode potentials, barometer corrections (CLARK and LUBS), 486
— — of phthalate, phosphate, and borate buffer mixtures (CLARK and LUBS), 479
— and tonometer (McCLENDON and MAGOON), 675
—, two compartment (McCLENDON and MAGOON), 669
Ion concentration of aluminium chloride solutions (MIYAKE), 20
— — — blood (GETTLER and BAKER), 221
Hydrogen—*continued:*
(McCLENDON and MAGOON), 672
— —, comparison solutions for colorimetric method (CLARK and LUBS), 479
— —, measurement of (SCHMIDT), 66
— —, optimum for liver autolysis (BRADLEY and TAYLOR), 264
— —, protein compounds, changes in, during formation of (SCHMIDT), 63
— — of serum (McCLENDON and MAGOON), 672
— — of stomach contents, indicator method for (McCLENDON and MAGOON), 680
Hydrolysis:
Yeast nucleic acid (LEVENE and JACOBS), 103
— — — with ammonia (JONES and GERMANN), 93
***p*-Hydroxybenzaldehyde:**
Ninhydrin reaction with ammonium salts, action on (HARDING and WARNEFORD), 330
β-Hydroxybutyric acid:
α-Aminocaproic acid, formation from (GREENWALD), 82
Hydroxyl ion:
Concentration, protein compounds, changes in formation of (SCHMIDT), 63
α-Hydroxypyridine:
Antineuritic properties (WILLIAMS), 439

α-Hydroxypyridine—*continued:*
Isomeric forms, antineuritic properties of (WILLIAMS), 440
Isomerism of (WILLIAMS), 442

β-Hydroxypyridine:
Antineuritic properties (WILLIAMS), 445

γ-Hydroxypyridine:
Antineuritic properties (WILLIAMS), 445

Hyperglycemia:
Anesthesia, production by (EPSTEIN and ASCHNER), 152
Epinephrine, calcium salts, influence of (UNDERHILL), 451
—, magnesium salts, influence of (UNDERHILL), 471
—, sodium salts, influence of (UNDERHILL), 456, 463
Magnesium sulfate, calcium chloride, influence of (UNDERHILL), 475
Nephrectomy, influence of (EPSTEIN and ASCHNER), 160
Stimuli, psychic and sensory, effect of, on hyperglycemia produced by cold (KRAMER and COFFIN), 423

Hypertrophy:
Protein relationships in (BRADLEY and TAYLOR), 279

I

2-Imidopseudouric acid:
(LEVENE and SENIOR), 619

4-Imidopseudouric acid:
(LEVENE and SENIOR), 618
Uric acid, conversion into (LEVENE and SENIOR), 621

Indicators:
Buffer value of blood, determination of, by (MCCLENDON and MAGOON), 679
Stomach contents, hydrogen ion concentration of, by (MCCLENDON and MAGOON), 680

Inosinic acid:
Tetany, rôle in (GREENWALD), 224

Inosite:
Intestine, absorption from (ANDERSON), 394
Metabolism of man, effect of (ANDERSON and BOSWORTH), 399
Nitrogen balance, effect on (ANDERSON and BOSWORTH), 403
Phosphorus balance, effect on (ANDERSON and BOSWORTH), 403
Utilization of, in the dog (ANDERSON), 391

Invertin:
Blood, effect of, on activity of (KURIYAMA), 541
— serum, content of, after injection of invertin (KURIYAMA), 539
— —, effect of, on activity of (KURIYAMA), 541
Sucrose, utilization of, effect on (KURIYAMA), 533
Urine content after injection of (KURIYAMA), 539

Involution:
Protein relationships in (BRADLEY and TAYLOR), 280
Irritability:
Electrical, of nerves, stimulation, relation to (LOEB and EWALD), 384
Isomerism:
Hydroxypyridines (WILLIAMS), 440

J

Jack bean:
Urease (MATEER and MARSHALL), 297

L

Lactic acid:
Protein metabolism, effect on (KOCHER), 573
Latent period:
Autolysis (BRADLEY and TAYLOR), 363
—, acids, effect of (BRADLEY and TAYLOR), 366
—, gelatin, effect of (BRADLEY and TAYLOR), 368
—, sodium bicarbonate, effect of (BRADLEY and TAYLOR), 369
Lauric acid:
Myristic acid, separation from (JACOBSON and HOLMES), 55
Salts, solubility of, in organic solvents (JACOBSON and HOLMES), 29
Lead salts:
Fatty acids, solubility of, in organic solvents (JACOBSON and HOLMES), 47
Lecithin:
Blood content (BLOOR), 585
Growth, influence on (ROBERTSON), 647
— of suckling mice, influence of, when fed to mother (ROBERTSON and CUTLER), 663
Pituitary body, content of (FENGER), 419
—, growth, influence on (ROBERTSON), 656
Levulose:
Ingestion, limit of (TAYLOR and HULTON), 175
Lipoids:
Blood, human, distribution in (BLOOR), 577
Lithium chloride:
Casein, rate of solution of, in sodium hydroxide, influence on (ROBERTSON and MIYAKE), 353
Lithium salts:
Fatty acids, solubility of, in organic solvents (JACOBSON and HOLMES), 33
Liver:
Autolysis, reaction, effect of (BRADLEY and TAYLOR), 261
Autolyzed, cholesterol content (MUELLER), 565
Lupinus albus:
Urease content (MATEER and MARSHALL), 298

γ-Lutidone:
Antineuritic properties (WILLIAMS), 445
Lysine:
Growth, rôle in (OSBORNE and MENDEL), 1
Maintenance, rôle in (OSBORNE and MENDEL), 1

M

Magnesium:
Nerve stimulation, inhibitory power on (LOEB and EWALD), 381
Magnesium lactate:
Blood sugar content, effect on (UNDERHILL), 472
Glycosuria, epinephrine, influence on (UNDERHILL), 472
Hyperglycemia, epinephrine, influence on (UNDERHILL), 472
Magnesium salts:
Fatty acids, solubility of, in organic solvents (JACOBSON and HOLMES), 39
Magnesium sulfate:
Anesthesia, hydrochloric acid, influence of (UNDERHILL), 477
—, sodium carbonate, influence of (UNDERHILL), 477
Blood sugar content, effect of (UNDERHILL), 474
Glycosuria, calcium chloride, influence of (UNDERHILL), 475
—, epinephrine, influence of (UNDERHILL), 474
Magnesium sulfate—*continued:*
Hyperglycemia, calcium chloride, influence on (UNDERHILL), 475
—, epinephrine, influence on (UNDERHILL), 474
Maintenance:
Amino-acid minimum for (OSBORNE and MENDEL), 1
Lysine, rôle of (OSBORNE and MENDEL), 1
Tryptophane, rôle of (OSBORNE and MENDEL), 1
Manganous carbonate:
Liver autolysis, effect on (BRADLEY and TAYLOR), 266
Meat scraps:
Growth, influence on (HART, MILLER, and MCCOLLUM), 247
Mercuric acetate:
Sugar in urine, use in determination of (KURIYAMA), 524
Mercuric chloride:
Blood proteins, precipitation of (GETTLER and BAKER), 214
Metabolism:
Carbohydrate (UNDERHILL), 447, 463, 471
Inosite, effect of, on metabolism in man (ANDERSON and BOSWORTH), 399
— utilization in dog (ANDERSON), 391
Protein, lactic acid, influence of (KOCHER), 573

Metabolism—*continued:*
—, pyruvic acid, influence of (KOCHER), 574
—, sparing action of carbohydrates on (KOCHER), 571
Methyl acetate:
Fatty acid salts, solubility of, in (JACOBSON and HOLMES), 37
Methyl alcohol:
Fatty acid salts, solubility of, in (JACOBSON and HOLMES), 34
Milk:
Albumin, serum, normal and sensitized, digestion by (HULTON), 168, 228
Powder, cottonseed meal and, growth on (RICHARDSON and GREEN), 313
Mucin:
Pig's stomach, conjugated sulfuric acid of (LEVENE and LÓPEZ-SUÁREZ), 511
Mucoitin sulfuric acid:
(LEVENE and LÓPEZ-SUÁREZ), 511
Glucosamine from (LEVENE and LÓPEZ-SUÁREZ), 514
Murexide:
Alloxan formation from, ninhydrin reaction, analogy to (HARDING and WARNEFORD), 320
Diketohydrindylidenediketohydrindamine, ammonium salt, relation to (HARDING and MACLEAN), 345
Muscle:
Creatine, determination of (BAUMANN and INGVALDSEN), 195
Non-protein substances, determination of (JANNEY), 177
Protein content (JANNEY), 183, 185
—, total, determination of (JANNEY), 177
Myristic acid:
Lauric acid, separation from (JACOBSON and HOLMES), 55
Salts, solubility of, in organic solvents (JACOBSON and HOLMES), 29

N

Naphthylureidocephalin:
(LEVENE and WEST), 519
Necrosis:
Protein relationships in (BRADLEY and TAYLOR), 278
Nephrectomy:
Blood sugar content, influence on (EPSTEIN and ASCHNER), 160
Nerves:
Chemical stimulation of (LOEB and EWALD), 377
Ninhydrin:
Alloxan, relation of (HARDING and MACLEAN), 344
Amides, reaction with (HARDING and MACLEAN), 337
Amines, reaction with (HARDING and MACLEAN), 337

Ninhydrin—*continued:*
Amino-acids, reaction with (HARDING and WARNEFORD), 319
Ammonia, reaction with (HARDING and WARNEFORD), 330
Ammonium salts, reaction with (HARDING and WARNEFORD), 319
— —, — —, pyridine, influence of (HARDING and WARNEFORD), 324
Reaction, analogy to murexide formation from alloxan (HARDING and WARNEFORD), 320
—, mechanism of (HARDING and WARNEFORD), 327
—, pyridine, effect of (HARDING and WARNEFORD), 324
(HARDING and MACLEAN), 338
—, reducing agents, action of (HARDING and WARNEFORD), 330

Nitrogen:
Blood content (GETTLER and BAKER), 213
Inosite, effect of, on balance of (ANDERSON and BOSWORTH), 403
Lactic acid, effect of, on excretion of (KOCHER), 573
Non-coagulable, live rautolysis, latent period in (BRADLEY and TAYLOR), 364
Non-colloidal, determination of (WELKER and FALLS), 567

Nitrogen—*continued:*
Non-protein, blood content (GETTLER and BAKER), 213
—, —, determination in (GETTLER and BAKER), 214
Partition, effect of substituting alcohol for sucrose (HAMMETT), 601
Plasma content (GETTLER and BAKER), 213
Pyruvic acid, effect of, on excretion of (KOCHER), 574
Serum content (GETTLER and BAKER), 213

Nitrous oxide:
Anesthesia, blood sugar, effect on (EPSTEIN and ASCHNER), 157
Ether and, anesthesia, blood sugar, influence on (EPSTEIN and ASCHNER), 157

Non-protein:
Muscle, determination in (JANNEY), 177
Nitrogen, blood content (GETTLER and BAKER), 211
Sera of animals and birds, content of (JEWETT), 21

Nor-leucine:
See α-Aminocaproic acid.

Nucleic acid:
Globin, compound with (SCHMIDT), 76

Nutrition:
Cottonseed meal in (RICHARDSON and GREEN), 307
Deficiency of wheat grain mixture in (HART, MILLER, and MCCOLLUM), 239

O

Oat diet:
Disease produced by, in guinea pigs and rabbits (FUNK), 409

Oil:
Wheat embryo, toxicity of (McCOLLUM, SIMMONDS, and PITZ), 109

P

Palmitic acid:
Salts, solubility of, in organic solvents (JACOBSON and HOLMES), 29

Panicularia nervata:
Hydrocyanic acid content of leaves (ALSBERG and BLACK), 136

Penetration:
Coefficient of (ROBERTSON and MIYAKE), 359

Pepsin:
Bence-Jones protein, digestion by (TAYLOR and MILLER), 293

Peptone:
Liver enzyme, digestion by (BRADLEY and TAYLOR), 269

Phaseolin:
Serum, normal and sensitized, digestion by (HULTON), 168, 228

Phaseolus aureus:
Urease content (MATEER and MARSHALL), 299

Phenol:
Determination of (MORRIS), 205

Phenylureidocephalin:
(LEVENE and WEST), 518

Phenylureidotetrahydrocephalin:
(LEVENE and WEST), 519

Phosphate buffer mixtures:
Hydrogen electrode potentials of (CLARK and LUBS), 479

Phosphoric acid:
Ammonium magnesium phosphate as form to weigh (JONES), 87

Liver autolysis, effect on (BRADLEY and TAYLOR), 263

Phosphorus:
Acid-soluble, of serum (GREENWALD), 431
Determination of (GERMANN), 192
Inosite, effect of, on balance of (ANDERSON and BOSWORTH), 403
Thymus nucleic acid, partition in (GERMANN), 189

Phthalate buffer mixtures:
Hydrogen electrode potentials of (CLARK and LUBS), 479

Phthalic acid:
Acid potassium salt as standard for hydrogen ion concentration (CLARK and LUBS), 506

***o*-Phthalic acid:**
Titration curve (CLARK and LUBS), 507

Physical:
Blood, analysis of normal (GETTLER and BAKER), 211

Picric acid:
Creatine, precipitation of (BAUMANN and INGVALDSEN), 197

Pituitary:
Adult and infant, composition and physiological activity (FENGER), 417
Chick, growth of, influence on (WULZEN), 630
Lecithin content (FENGER), 419
—, growth, influence on (ROBERTSON), 656
Planarian worms, growth of, influence on (WULZEN), 625
Reproduction, effect on (WULZEN), 625
Uterine-contracting principle (FENGER), 421

Placenta:
Ferments, specific proteoclastic, formation of, by introduction of (HULTON), 227
Protein, digestion by normal and sensitized serum (HULTON), 228

Planarian worms:
Pituitary body, effect of, on growth and fission of (WULZEN), 625

Plants:
Cyanogenesis in (VIEHOEVER, JOHNS, and ALSBERG), 141
Hydrocyanic acid, recovery of, from (VIEHOEVER, JOHNS, and ALSBERG), 146

Plants—*continued:*
Tissue, cyanogenetic, action of hydrocyanic acid on (ALSBERG and BLACK), 136
—, hydrocyanic acid, separation of, from (ALSBERG and BLACK), 133

Plasma:
Carbon dioxide capacity (GETTLER and BAKER), 219
Nitrogen content (GETTLER and BAKER), 213

Potassium chloride:
Casein, rate of solution of, in sodium hydroxide, influence on (ROBERTSON and MIYAKE), 355

Potassium cyanide:
Plant tissues, recovery from (ALSBERG and BLACK), 133

Potassium dihydrogen phosphate:
Liver autolysis, effect on (BRADLEY and TAYLOR), 263

Potato juice:
Oat disease in rabbits, effect on (FUNK), 413

Potentials:
Diffusion (CLARK and LUBS), 483
Hydrogen electrode (CLARK and LUBS), 479

Protamine:
Serum, normal and sensitized, digestion by (HULTON), 168, 228

Protein:
Alcohol, precipitation by (JANNEY), 178
Bence-Jones (TAYLOR and MILLER), 281
—, anaphylaxis (TAYLOR and MILLER), 290
—, digestibility (TAYLOR and HULTON), 293
—, preparation of (TAYLOR and HULTON), 288
—, serum, normal and sensitized, digestion by (HULTON), 168, 228
—, toxicity of (TAYLOR and MILLER), 293
Body, normal hydrolysis of (HULTON), 170
Compounds, formation of, changes of H^+ and OH^- concentrations during (SCHMIDT), 63
Cottonseed flour, value of, for growth (RICHARDSON and GREEN), 310
Factor (JANNEY), 185
Foreign, ferments, specific proteoclastic, formation of, after parenteral injection of (HULTON), 163
Liver cells, relationships in (BRADLEY and TAYLOR), 276
—, reaction, effect of (BRADLEY and TAYLOR), 275
Mercuric chloride, precipitation by (GETTLER and BAKER), 214
Metabolism, carbohydrate, sparing action of (KOCHER), 571
Protein—*continued:*
—, lactic acid, effect of, on (KOCHER), 573
—, pyruvic acid, effect of, on (KOCHER), 574
Muscle, content of (JANNEY), 183, 185
—, determination in (JANNEY), 177
Placenta, serum, normal and sensitized, digestion by (HULTON), 228
Relationships in autolysis (BRADLEY and TAYLOR), 277
Serum, digestion by (HULTON), 168, 228
Sparing action of alcohol (HAMMETT), 604
Total, sera of animals and birds, content of (JEWETT), 21
Wheat embryo, value of, for growth (McCOLLUM, SIMMONDS, and PITZ), 107
Proteinuria:
Bence-Jones (TAYLOR and MILLER), 281
Prunus virginiana:
Hydrocyanic acid content of leaves (ALSBERG and BLACK), 136
Pyridine:
Ninhydrin reaction, effect on (HARDING and WARNEFORD), 324
(HARDING and MACLEAN), 338
Pyruvic acid:
Protein metabolism, effect on (KOCHER), 574

R

Reaction:
Latent period in autolysis, effect on (Bradley and Taylor), 368
Liver autolysis, effect on (Bradley and Taylor), 261

Reducing agents:
Ninhydrin reaction, effect on (Harding and Warneford), 330

Refractive index:
Blood (Gettler and Baker), 221

Reproduction:
Cottonseed meal, influence on (Richardson and Green), 314
Pituitary, effect of (Wulzen), 625

Respiratory quotient:
Inosite, effect of (Anderson), 394

Rice plant:
Growth, hydrochloric acid, influence of, on (Miyake), 26
—, toxic action of aluminium salts (Miyake), 25

S

Salts:
Bence-Jones urine, reactions of (Taylor and Miller), 284
Growth, effect on (Hart, Miller, and McCollum), 245
Lauric acid, solubility of, in organic solvents (Jacobson and Holmes), 29
Liver autolysis, effect on (Bradley and Taylor), 265

Salts—*continued:*
Myristic acid, solubility of, in organic solvents (Jacobson and Holmes), 29
Palmitic acid, solubility of, in organic solvents (Jacobson and Holmes), 29
Stearic acid, solubility of, in organic solvents (Jacobson and Holmes), 29

Serum:
Albumin content (Jewett), 21
Animals, non-protein content (Jewett), 21
Birds, non-protein content (Jewett), 21
Blood, invertin content after injection of invertin (Kuriyama), 539
—, non-colloidal nitrogen determination (Welker and Falls), 567
Buffer value, charts for (McClendon and Magoon), 678
Carbon dioxide tension, buffer value, relation to (McClendon and Magoon), 679
Colloids, precipitation of, with aluminium hydroxide cream (Welker and Falls), 567
Conductivity (Gettler and Baker), 220
Dextrose, action on (Kuriyama), 538
Globulin content (Jewett), 21

Serum—*continued:*
Hydrogen ion concentration (McClendon and Magoon), 672
Intestinal sucrase, effect on activity of (Kuriyama), 541
Invertin activity, effect on (Kuriyama), 541
Nitrogen content (Gettler and Baker), 213
Non-protein content (Jewett), 21
Phosphorus, acid-soluble, of (Greenwald), 431
Protein, composition and blood relationships of animals (Jewett), 21
— content (Jewett), 21
—, digestion of, by normal and sensitized (Hulton), 168, 228

Silver salts:
Fatty acids, solubility of, in organic solvents (Jacobson and Holmes), 51

Sodium benzoate:
Uric acid excretion in man, influence on (Lewis and Karr), 13

Sodium bicarbonate:
Latent period in liver autolysis, effect on (Bradley and Taylor), 369
Liver autolysis, effect on (Bradley and Taylor), 267

Sodium carbonate:
Blood sugar content, effect on (Underhill), 463
Glycosuria, epinephrine, influence on (Underhill), 463
Hyperglycemia, epinephrine, influence on (Underhill), 463
Magnesium sulfate anesthesia, influence on (Underhill), 477

Sodium chloride:
Casein, rate of solution in sodium hydroxide, influence on (Robertson and Miyake), 354

Sodium hydroxide:
Casein, rate of solution in, alkali and alkaline earth salts, influence of, on (Robertson and Miyake), 351
Liver autolysis, effect on (Bradley and Taylor), 268

Sodium oxalate:
Blood sugar content influence on (Underhill), 456
Glycosuria, epinephrine, influence on (Underhill), 456

Sodium phosphate:
Blood sugar content, influence on (Underhill), 456
Glycosuria, epinephrine, influence on (Underhill), 456
Hyperglycemia, epinephrine, influence on (Underhill), 456

Sodium salts:
Nerves, stimulation of (LOEB and EWALD), 378
Stimulating effect, rôle of sodium chloride in (LOEB and EWALD), 382
Soil fertility:
Determination, logical method of (MIYAKE), 28
Solids:
Blood content (GETTLER and BAKER), 213
Soy bean:
Globulin, serum, normal and sensitized, digestion by (HULTON), 168, 228
Urease content (MATEER and MARSHALL), 298
Specific gravity:
Blood (GETTLER and BAKER), 221
Spinal cord:
Pathological condition from wheat feeding (HART, MILLER, and MCCOLLUM), 242
Starvation:
Protein relationships in (BRADLEY and TAYLOR), 278
Stearic acid:
Salts, solubility of, in organic solvents (JACOBSON and HOLMES), 29
Stimulation:
Chemical, of nerves (LOEB and EWALD), 377
—, relation to electrical stimulation (LOEB and EWALD), 386
Stimulation—*continued:*
Latent period of, effect of calcium on (LOEB and EWALD), 384
Stimuli:
Psychic and sensory, effect of, on hyperglycemia produced by cold (KRAMER and COFFIN), 423
Stomach content:
Hydrogen ion concentration, indicator method for (MCCLENDON and MAGOON), 680
Strontium chloride:
Casein, rate of solution of, in sodium hydroxide, effect on (ROBERTSON and MIYAKE), 356
Substratum:
Latent period in liver autolysis, effect on (BRADLEY and TAYLOR), 368
Sucrase:
Intestinal, blood, effect of, on activity of (KURIYAMA), 541
—, serum, effect of, on activity of (KURIYAMA), 541
Sucrose:
Blood, inverting power of, after parenteral injection of (KURIYAMA), 534
Nitrogen partition, effect on, of substituting alcohol for (HAMMETT), 601
Utilization (KURIYAMA), 521
—, invertin, effect of, on (KURIYAMA), 533

Sugar:
Blood. *See* Blood sugar.
Urine content, epinephrine, influence of (UNDERHILL), 450
Sulfuric acid:
Conjugated, of mucin of pig's stomach (LEVENE and LÓPEZ-SUÁREZ), 511
Surgical procedures:
Blood sugar content, effect on (EPSTEIN and ASCHNER), 151
Sword bean:
Urease content (MATEER and MARSHALL), 297

T

Tall red top:
Cyanogenesis in (VIEHOEVER, JOHNS, and ALSBERG), 141
Taurocholic acid:
Globin, compound with (SCHMIDT), 76
Testicles:
Cholesterol, effect of, on growth of (ROBERTSON), 643
Tetany:
Inosinic acid, rôle of (GREENWALD), 224
Parathyroidectomized dogs (GREENWALD), 223
Tetraethylammonium chloride:
Nerve muscles, stimulation of (LOEB and EWALD), 382
Stimulating action, rôle of sodium chloride in (LOEB and EWALD), 379
Thymus nucleic acid:
Phosphorus partition in (GERMANN), 189
Tissues:
Cholesterol esters, action on (MUELLER), 561
Tonometer:
Hydrogen electrode and (MCCLENDON and MAGOON), 675
Toxicity:
Aluminium chloride for rice plant (MIYAKE), 23
Bence-Jones protein (TAYLOR and MILLER), 293
Cottonseed meal (RICHARDSON and GREEN), 314
Wheat for swine (HART, MILLER, and MCCOLLUM), 244
Tridens flavus:
Cyanogenesis in (VIEHOEVER, JOHNS, and ALSBERG), 141
Hydrocyanic acid content (VIEHOEVER, JOHNS, and ALSBERG), 141
2,3,4-Trihydroxypyridine:
Antineuritic properties (WILLIAMS), 439
2,4,6-Trihydroxypyridine:
Antineuritic properties (WILLIAMS), 439
Triketohydrindene hydrate:
See Ninhydrin.
Triphosphonucleinsäure:
Guanylic acid, identity with (JONES and GERMANN), 99
Trypsin:
Bence-Jones protein, digestion by (TAYLOR and MILLER), 293
Tryptophane:
Growth, rôle in (OSBORNE and MENDEL), 1

Tryptophane—*continued:*
Maintenance, rôle in (Osborne and Mendel), 1

U

Urea:
Blood content (Gettler and Baker), 215
Urease:
Bean extracts, relative activity (Mateer and Marshall), 299
Jack bean, preparation from (Mateer and Marshall), 303
— —, specificity of (Mateer and Marshall), 301
Uric acid:
Blood content (Gettler and Baker), 215
Determination of (Morris), 205
—, colorimetric, effect of sodium hippurate (Lewis and Karr), 14
Excretion in man after sodium benzoate ingestion (Lewis and Karr), 13
— — — — sodium hippurate (Lewis and Karr), 19
Inosite, effect of, on excretion (Anderson and Bosworth), 404
Zinc salt (Morris), 205
Urine:
Bence-Jones, acids, behavior towards (Taylor and Miller), 285
— —, anaphylaxis (Taylor and Miller), 292
Urine—*continued:*
— — protein, heat reactions of (Taylor and Miller), 282
— — —-free, preparation of (Taylor and Miller), 290
— —, salt reactions of (Taylor and Miller), 284
Formaldehyde, determination of (Collins and Hanzlik), 234
Hexamethylenetetramine, determination of (Collins and Hanzlik), 234
Inosite, excretion of, in man (Anderson and Bosworth), 402
Invertin content after injection of invertin (Kuriyama), 539
Sugar content, epinephrine, influence of (Underhill), 450
Utilization:
Inosite by dog (Anderson), 391
— by man (Anderson and Bosworth), 399
Sucrose (Kuriyama), 521
—, invertin, effect of (Kuriyama), 533

V

Vicine:
Constitution (Levene and Senior), 607
Vitamines:
Chemical nature of (Williams), 437

W

Water:
Fatty acid salts, solubility of, in (JACOBSON and HOLMES), 35

Wheat:
Embryo, dietary deficiencies (MCCOLLUM, SIMMONDS, and PITZ), 105
—, ether-extracted, nutritive value of (MCCOLLUM, SIMMONDS, and PITZ), 109
—, feeding experiments with (MCCOLLUM, SIMMONDS and PITZ), 107
—, oil, toxicity of (MCCOLLUM, SIMMONDS, and PITZ), 109
—, proteins, value of, for growth (MCCOLLUM, SIMMONDS, and PITZ), 108
Nutritive deficiencies (HART, MILLER, and MCCOLLUM), 239
Toxic substance of, swine, susceptibility of, towards (HART, MILLER, and MCCOLLUM), 244

White lupine:
Urease content (MATEER and MARSHALL), 298

Y

Yeast:
Oat disease in rabbits, effect on (FUNK), 412

Yeast nucleic acid:
Adenine-uracil dinucleotide, preparation of (JONES and GERMANN), 99
Ammonia, hydrolysis with (JONES and GERMANN), 93
(LEVENE and JACOBS), 103

Z

Zein:
Feeding experiments with (OSBORNE and MENDEL), 4

Zinc oxide:
Liver autolysis, effect on (BRADLEY and TAYLOR), 267

Zinc salts:
Uric acid (MORRIS), 205

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